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By virtue of a direction under Section 30 of the patents Act 1977, the application is proceeding in the name of,

AMURA THERAPEUTICS LIMITED,
Incorporated in the United Kingdom,
Incenta House,
Horizon Park,
Barton Road,
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[ADP No. 08535981001]

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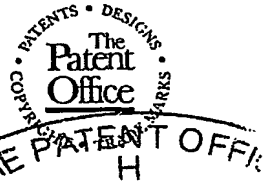

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08 APR 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Incenta Limited
St John's Innovation Centre
Cowley Road
Cambridge
CB4 0WS

02-01-03.

SECTION 20(1) 77 ACT) APPLICATION FILED

08062358001
Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

Incorporated in England

4. Title of the invention

Organic Molecules

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Andrew Sheard, Patent Attorney
PO Box 521
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Patents ADP number *(if you know it)* 08062671001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
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Number of earlier application	Date of filing *(day / month / year)*
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Description	41
Claim(s)	9
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Priority documents	Not filed
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	Not filed
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Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature A G Sheard Date 8 April 2002

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ORGANIC MOLECULES

THE PRESENT INVENTION relates to protein constructs in which a plurality of small molecules are attached to a carrier protein. In particular, the invention relates to high loading soluble protein constructs in which the small molecules, or epitopes, are linked to the carrier protein *via* a linker which is selected such that the charge pattern at the surface of the loaded carrier protein closely resembles that of the unloaded carrier protein.

Background

Small molecules and peptides identified as epitopes for vaccine development usually require conjugation to carrier proteins to provide a construct with which to provoke an immune response to the low molecular weight immunogen *in vivo*. This is illustrated in Scheme 1 below where an epitope (**1**) is reacted with a conjugate (**2**) and a carrier protein (**3**) to give a construct (**4**). An ideal vaccine construct would contain a high surface coverage of conjugated epitope on the carrier protein, whilst retaining high aqueous solubility. Additionally, the linkage created between the carrier protein and epitope ideally would be immunogenically inert and not involve residues or functionalities critical to epitope recognition (Briand. J.P., Muller, S. and Van Regenmortal, M.H.V. *J. Immunol. Methods* **78**, 59-69, 1985). Presently, the most commonly used methods of conjugation in the preparation of experimental vaccines involve chemically non-specific reactions with glutaraldehyde (Avrameas, S. and Ternynck, T. *Immunochemistry* **6**, 53, 1969; Korn, A.H., Feairheller, S.H. and Filachione, E.M. *J. Mol. Biol.* **65**, 525, 1972; Reichlin, M. in: *Methods in Enzymology*, vol. 70, eds. Van Vunakis, H. and Langone, J.J. [Academic Press, New York] pp. 159-165, 1980), carbodiimides (Goodfriend, T.L., Levine, L. and Fasman, G.D. *Science* **144**, 1344, 1964; Bauminger, S. and Wilchek, M. in: *Methods in Enzymology*, vol. 70, eds. Van Vunakis, H. and Langone, J.J. [Academic Press, New York] pp. 151-159, 1980), bis-diazotized benzidine (BDB) (Gordan, J., Rose, B. and Sehon, A.H. *J. Exp.*

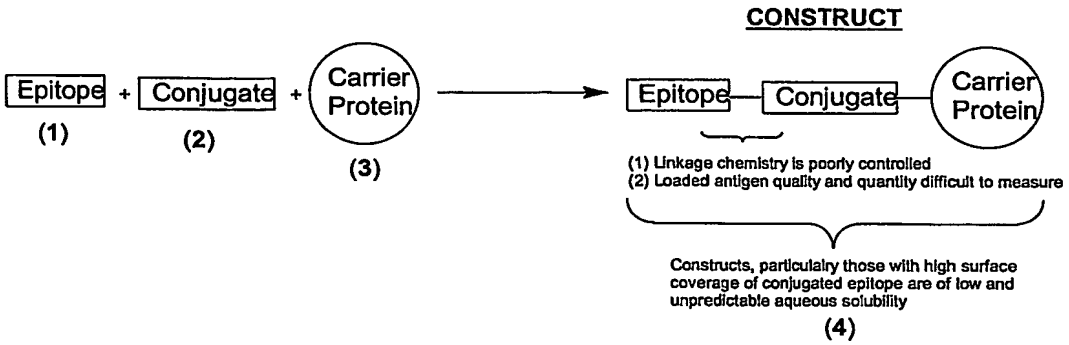
Med. **108**, 37, 1958) or utilise maleimide derivatives that rely on the presence of a thiol moiety (Liu, F.-T., Zinnecker, M., Hamaoka, T. and Katz, D.H. *Biochemistry* **18**, 690, 1979; Yoshitake, S., Yamada, Y., Ishikawa, E. and Masseyeff, R. *Eur. J. Biochem.* **101**, 395-399, 1979; Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T.M., Sutcliffe, J.G. and Lerner, R.A. *Cell* **28**, 477, 1982).

Since the chemical nature of the epitope, particularly in the field of peptide epitopes, is a rapidly advancing discipline, many of the above techniques are no longer fully compatible with the more advanced epitope chemistries. The introduction of improved structural characterisation and design elements such as the use of disulfide constrained peptide loops as structural mimics of the epitope in its native environment, require specific and acutely controlled methods of conjugation to ensure the chemical integrity of the loaded epitope. Here, existing conjugation methods suffer from a number of experimental difficulties (Scheme 1):

(a) Qualitative and quantitative assessment of loaded epitope from the construct, the sometimes chemically sensitive epitope having proceeded through a number of chemical processes, is problematic (Briand. J.P., Muller, S. and Van Regenmortal, M.H.V. *J. Immunol. Methods* **78**, 59-69, 1985).

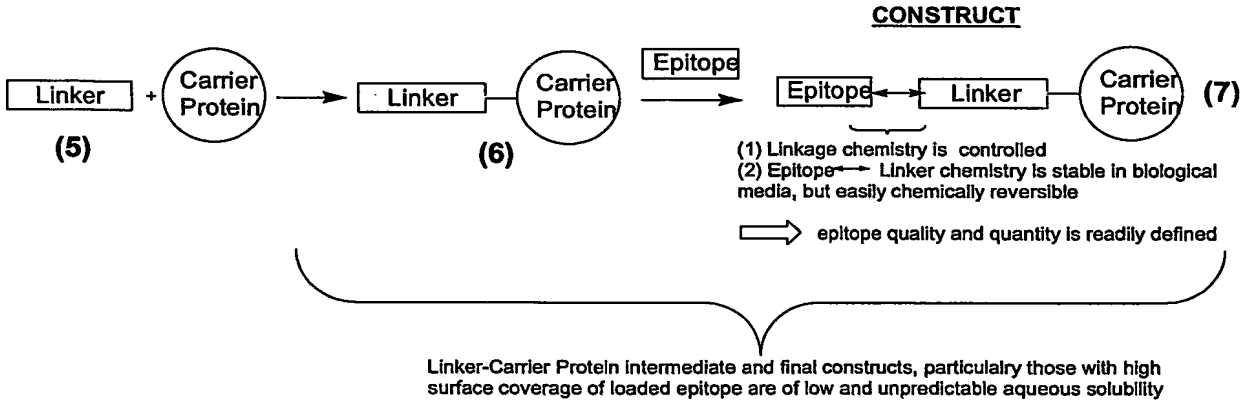
(b) Generally, as the surface loading of carrier proteins with the conjugated epitope increases, low and unpredictable solubility of the construct is observed (Qamar, S., Islam, M. and Tayyab, S. *J. Biochem.* **114**, 786-792, 1993).

(c) Introduction of disulfide-bridged peptides is complicated by the presence of the thiol functionality required when using maleimide-based conjugation and may lead to disruption of the disulfide bond.



Scheme 1. Traditional conjugation of an antigen to a carrier protein

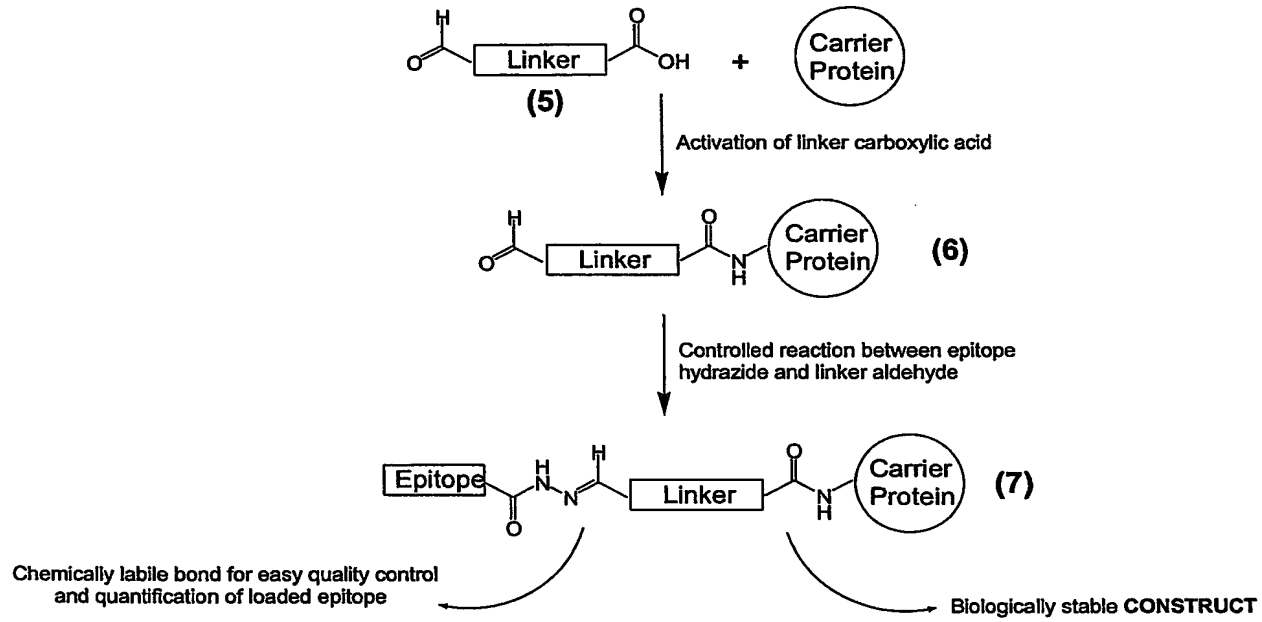
A number of the above issues have begun to be addressed in a new generation of construct detailed in WO-A-0145745, which describes the invention of a process that allows the controlled linkage of a peptidic epitope to a carrier protein. The process provides a construct (7) from which the qualitative and quantitative assessment of epitope loading can be determined by simple chemical means as illustrated in Schemes 2 & 3.



Scheme 2. WO-A-0145745 controlled conjugation of an epitope to a carrier protein

WO-A-0145745 describes a chemical linker (5) that contains a carboxylic acid and an aldehyde functionality. The carboxylic acid provides a point of attachment to the carrier protein by the formation of a secondary amide bond between the linker and the carrier protein accessible surface lysine residues – a process that yields an intermediate Linker-Carrier Protein (6). The aldehyde functionality provides a point of attachment

to a peptidic epitope in a controlled and chemically reversible manner. Since the peptidic epitope itself may contain many chemically reactive functionalities (amino acid residue side chains containing amine, carboxylic acid, thiol, alcohol, imidazole, indole), controlled reaction to **(6)** is achieved through the chemoselective reaction of **(6)** with a hydrazide function, introduced into the epitope during synthesis (Scheme 3).



Scheme 3. WO-A-0145745 controlled and reversible conjugation of an epitope to a carrier protein

The hydrazide, being a weak base, forms a stable acyl-hydrazone bond with the aldehyde functionality in **(6)** at acidic pH. At this low pH, basic side chain nucleophiles on the epitope are protonated and excluded from the conjugation reaction (Jencks, W.P. *J. Am. Chem. Soc.* **81,** 475-481, 1959; Reeves, R.L. in: *The Chemistry of the Carbonyl Group*, ed. Patai, S. (Interscience, London) pp. 600-614, 1966). Hydrazone formation has previously been employed in conjugation reactions *via* C-terminal hydrazides and N-terminal aldehydes that are traditionally generated by sodium metaperiodate mediated oxidation of an N-terminal serine residue within the specific proteins and peptides (King, T.P., Zhao, S.W. and Lam, T. *Biochemistry* **25,**

5774-9, 1986; Rose, K., Vilaseca, L.A., Werlen, R., Meunier, A., Fisch, I., Jones, R.M. and Offord, R.E. *Bioconj. Chem.* **2**, 154-159, 1991; Gaertner, H.F., Rose, K., Cotton, R., Timms, D., Camble, R. and Offord, R.E. *Bioconj. Chem.* **3**, 262-268, 1992).

The process described in WO-A-0145745 offers a clear advance compared with previous methodologies due to the controlled nature of the conjugation procedure. This process allows a high level of construct quality control to be achieved, through chemical release and analytical characterisation of the intact epitope **(1)**.

WO-A-0145745 has provided an impressive advance beyond previous methods. However, the whole question of construct solubility, an equally important consideration for the raising of antibodies and vaccination has not been addressed.

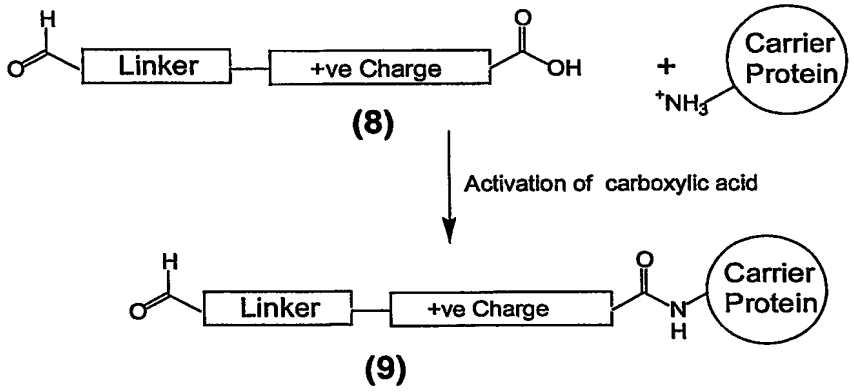
Widely used carrier proteins such as bovine serum albumin (BSA), ovalbumin and keyhole limpet haemocyanin (KLH) have a finely balanced surface distribution of charge. Conjugation of a linker/epitope preparation to these and other proteins disrupts the balance and distribution of charge within the protein and leads to an overall change in its isoelectric point (PI) and often results in conjugates with poor aqueous solubility at a relevant pH. As detailed in Scheme 3, each linker unit reacts with an accessible surface lysine residue to form a secondary amide bond, thus removing a positive charge from the carrier protein surface. As the surface coverage increases, a concomitant increase in unbalanced surface negative charge occurs, along with increasing steric hindrance within the construct (Ansari, A.A., Kidwai, S.A. and Salahuddin, A. *J. Biol. Chem.* **250**, 1625-32, 1975). Conformational change is brought about as the levels of acylation increase, as shown in a previous study of the succinylation of BSA, in which a change of Stokes radius from 3.7 to 6.3nm was observed upon 87% succinylation (Tayyab, S. and Qasim, M.A. *Biochim. Biophys. Acta* **913**, 359-367, 1987). This may eventually lead to a destabilisation of the carrier protein tertiary structure and precipitation of the construct. Furthermore, because of

the increase in net negative charge, the PI of the modified protein is reduced. Thus, as the pH of the solvent is lowered and approaches the new isoelectric point, the tendency of proteins to precipitate in the acidic media then becomes more likely (Shaw, K.L., Grimsley, G.R., Yakovlev, G.I., Makarov, A.A. and Pace, C.N. *Prot. Sci.* **10**, 1206-15, 2001). This is particularly relevant to our preferred method of conjugation, since the final hydrazone bond formation between epitope and linker-carrier protein **(6)** is performed at acidic pH (down to pH 2.1) (Rose, K., Zeng, W., Regamey, P.O., Chernushevich, I.V., Standing, K.G. and Gaertner, H.F. *Bioconj. Chem.* **7**, 552-556, 1996). Indeed in the case of WO-A-0145745 precipitation of the linker-carrier protein intermediate **(6)** was observed prior to attempted loading of epitope.

A major cause of construct insolubility at high surface coverage may be due to the build-up of unbalanced surface charge upon loading of epitope-linker. Modification of groups contributing negative charge may result in a net increase in the isoelectric point, whereas alteration of the positive charge bearing functions may result in net decrease in the isoelectric point of the protein. As the relationship between solubility and pH is a function of the isoelectric point of the protein, the ability to replace either positive or negative charge lost through chemical modification, provides an efficient way of controlling/improving the aqueous solubility of highly modified proteins. This necessitates the design and construction of a charge-balanced linker **(8)**. Restoration of charge balance within the construct is conceptually simple and may be brought about, in our case, through the inclusion of an amine to replace that substituted during the conjugation (a negative charge may be replaced through the inclusion of functional groups containing a proton that readily dissociates *e.g.* hydroxyl or carboxylic acid moieties). However, this solution is not trivial, since the chemistry involved in the initial linker-carrier protein formation involves amide bond formation and as such, any amine functionality within the linker would need to be protected prior to and unmasked following the acylation of the carrier protein with the linker. A more pragmatic approach to charge-restoration would be through the introduction of a

quaternary ammonium group. The quaternary nitrogen bears the positive charge, while remaining inert to further acylation.

The effect of quaternization on the solubility of proteins is well documented (Yamada, H., Seno, M., Kobayashi, A., Moriyama, T., Kosaka, M., Ito, Y. and Imoto, T. *J. Biochem.* **116**, 852-857, 1994) and has also been employed to improve solubility of synthetic polymers and macromolecular constructs (Ishizu, K. and Kitano, H. *J. Colloid Interface Sci.* **229**, 165-167, 2000; Thanou, M.M., Kotze, A.F., Scharringhausen, T., Luessen, H.L. de Boer, A.G., Verhoef, J.C. and Junginger, H.E. *J. Contr. Release* **64**, 15-25, 2000). Attachment of such a linker to the carrier protein will lead to a high loading and soluble, positive charge-balanced linker-carrier protein **(9)** (Scheme 4). In addition to the improved solubility characteristics, construct **(9)** could also be prepared as a core stock reagent, enabling uniform preparation of vaccine candidates and allowing a more precise comparison of different immunogens.



Scheme 4. High loading and soluble positive charge-balanced linker- carrier protein conjugates

The design and preparation of a robust positive charge balanced linker **(8)** is a surprisingly demanding task, within which many interlinked properties need to be considered:-

(a) Ideally, the positive charge in **(8)** should be in close proximity to the protein surface lysine charge that is removed upon coupling, and provide a centre of comparable pKa.

(b) The preparation of **(8)** should be smooth and reproducible.

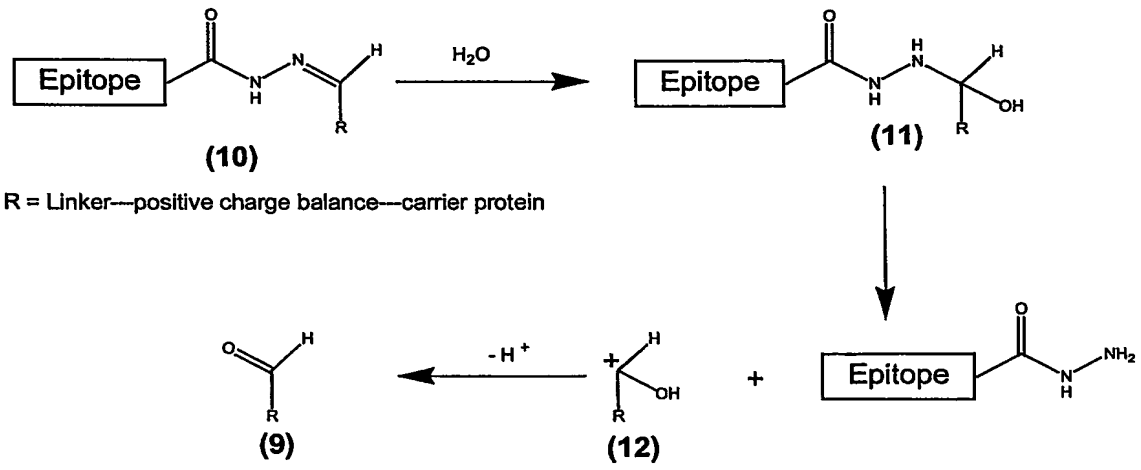
(c) Carboxylic acid activation of **(8)** prior to addition to the carrier protein should proceed smoothly with minimal interference from the positive charge.

(d) Formation of the secondary amide bond between **(8)** and the carrier protein should proceed smoothly with minimal interference from the positive charge.

(e) Formation of the acyl hydrazone bond between the epitope-hydrazide and positive charge balanced linker-carrier protein **(9)** should proceed smoothly with minimal interference from the positive charge.

(f) The reversible acid lability of the acyl hydrazone bond between the epitope-hydrazide and **(9)** requires the linker element of **(8)** to be an electron-rich aromatic moiety.

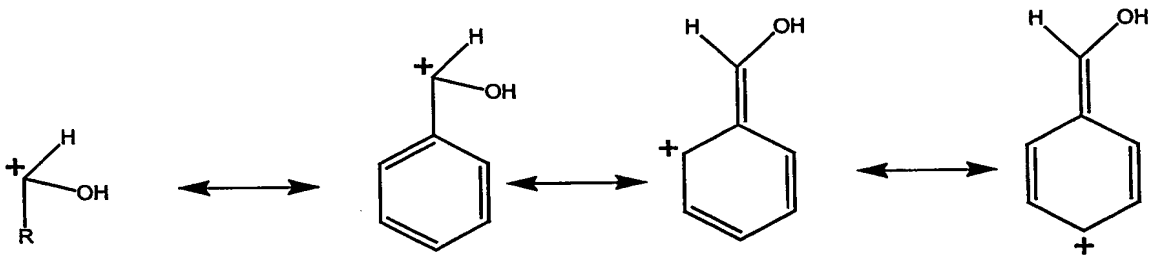
(g) The reversible acid lability of the acyl hydrazone bond between the epitope-hydrazide and **(9)** should not be adversely altered by the presence of the positive charge.



Scheme 5. Possible mechanism of hydrolysis of epitope hydrazone-linker construct **(9)**

The most critical theoretical design element relevant to the properties described in (a) → (g) is the retention of the reversible acid lability of the epitope acyl hydrazone bond in construct **(10)**, this being the key property allowing analytical analysis of the loaded epitope hydrazide on construct **(9)** (Scheme 5).

A possible mechanism for the hydrolysis of **(10)** proceeds through the addition of water to the carbon-nitrogen double bond giving **(11)**. This would be followed by elimination of the epitope acyl hydrazide (hence readily available for analysis) and formation of an intermediate carbocation **(12)**, then loss of a proton to give construct **(9)**. The ease of this hydrolytic process will in part depend upon the resonance stabilisation of carbocation **(12)**.



(12)

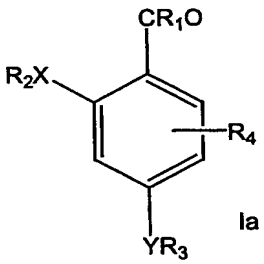
R = aromatic

Scheme 6. Resonance stabilisation of carbocation **(12)**

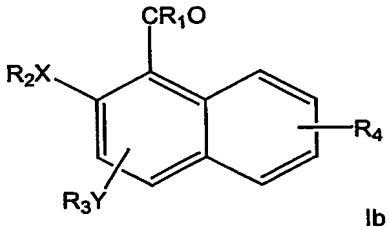
Resonance stabilisation of carbocation **(12)** is most readily achieved through an aromatic ring, preferably an electron-rich aromatic ring and more preferably a ring that contains electron-donating substituents situated ortho and para (see Scheme 6). Many examples exist in the literature describing the relationship between acid lability and substitution stereoelectronics for aromatic systems (e.g. see Johnson, T., Quibell, M. and Sheppard, R. C. *J. Pept. Sci.* **1**, 11-25, 1995). Ortho and para alkoxy-type substituents are required on linker **(8)** so that the *epitope*-linker hydrazone bond is labile to 1N HCl or trifluoroacetic acid (TFA) i.e. relatively mild conditions that do

not adversely affect peptidic *epitopes* and will allow easy hydrolysis and representative analysis of *epitope* from *construct* (9).

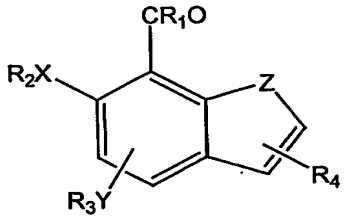
Thus, accordingly, the first aspect of the invention provides a positive charge-balanced linker according to general formulae (Ia to Ie):



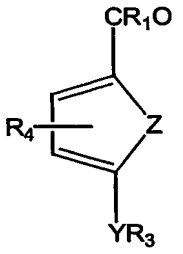
Ia



Ib



Ic



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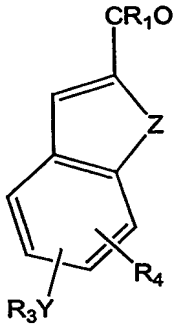


Ie

General formulae (I)

wherein:

X = O or S;

Y is O, S or CHR_4;

Z is O or S;

R_1 is H or C_{1-7} alkyl;

R_2 is H or C_{1-7} alkyl;

R_3 is C_{1-7} alkyl-L_1-R_5-L_2-R_6, C_{3-10} cycloalkyl-L_1-R_5-L_2-R_6 or Ar-C_{0-7} alkyl-L_1-R_5-L_2-R_6;

R_4 is H or C_{1-7} alkyl at any vacant position on the aromatic ring;

each of L_1 and L_2 is absent or a suitable linker such as CONH;

R_5 is C_{1-7} alkyl or C_{3-6}-cycloalkyl or Ar-C_{0-7}-alkyl containing a trisubstituted nitrogen atom $NR_7R_8R_9$, or is a quaternary nitrogen atom $N^+R_7R_8R_9R_{10}$ such that the substituent R_5 contains a positively charged nitrogen atom;

R_5 is C_{1-7} alkyl, C_{3-10} cycloalkyl or Ar-C_{0-7} alkyl each of which is substituted with a trisubstituted nitrogen atom $NR_7R_8R_9$ or a quaternary nitrogen atom $N^+R_7R_8R_9R_{10}$, such that R_5 contains a positive charge;

each of R_7, R_8, R_9 and R_{10} is independently hydrogen, C_{1-7} alkyl, C_{3-10} cycloalkyl or Ar-C_{0-7} alkyl, or any two or more of R_7, R_8, R_9 and R_{10} together form an alicyclic or arylalicyclic ring system;

R_6 is C_{1-7} alkyl-COOH, C_{3-10} cycloalkyl-COOH or Ar-C_{0-7} alkyl-COOH;

or a salt, hydrate, solvate, complex or prodrug thereof.

Compounds of Formula I can be reacted with a carrier protein to give a derivatised protein in which the surface charge pattern is substantially the same as that of the original carrier protein.

In the present specification, the term 'heteroatom' defines oxygen (O), sulphur (S) and nitrogen (N);

'Halogen' defines fluorine (F), chlorine (Cl), and bromine (Br).

'C_{1-7}-alkyl' as applied herein is meant to include stable straight or branched aliphatic carbon chains containing one to seven carbon atoms such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, t-butyl, pentyl, isopentyl, hexyl, heptyl and any simple isomers thereof. Additionally, any C_{1-7}-alkyl may optionally be substituted at any point by one, two or three halogen atoms (as defined above) for example to give a trifluoromethyl substituent. Furthermore, C_{1-7}-alkyl may contain one or more heteroatoms (as defined above) for example to give ethers, thioethers, sulphones, sulphonamides, substituted amines, amidines, guanidines, carboxylic acids, carboxamides. A heteroatom or halogen is only present when C_{1-7}-alkyl contains a minimum of two carbon atoms.

'C_{3-10}-cycloalkyl' as applied herein is meant to include any variation of 'C_{1-7}-alkyl', which additionally contains a 3 to 6 membered carbocyclic ring such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl. The carbocyclic ring may optionally be substituted with one or more halogens (as defined above) or heteroatoms (as defined above) for example to give a tetrahydrofuran, pyrrolidine, piperidine, piperazine or morpholine substituent.

'Ar-C_{0-7}-alkyl' as applied herein is meant to include any variation of C_{1-7}-alkyl which additionally contains an aromatic ring moiety 'Ar'. The aromatic ring moiety Ar can be a stable 5 or 6-membered monocyclic or a stable 9 or 10 membered bicyclic ring which is unsaturated. The aromatic ring moiety Ar may be additionally substituted by any variation of C_{1-7}-alkyl. When C = 0 in the substituent Ar-C_{0-7}-alkyl, the substituent is simply the aromatic ring moiety Ar.

The present invention includes all salts, hydrates, solvates, complexes and prodrugs of the compounds of this invention. Additionally, the present invention includes all isomers of stereochemical centres.

In the context of the present invention, the term "epitope" refers to a molecule which is capable of binding specifically to a biological molecule such as an antibody, antigen or cell surface receptor. The epitope may be a fragment, for example an antigenic determinant, derived from a protein or peptide molecule or a variant or analogue of such a molecule. Examples of epitopes which can be used with this method include oxytocin and analogues thereof. In the present invention, epitopes will be derivatised in order to allow them to react with the linker of General formula I. Suitable derivatives include hydrazide analogues. Where the epitope is a peptide, derivatisation may be achieved by reaction of a lysine side chain nitrogen.

"Carrier protein" refers to a proteinaceous molecule containing a plurality of active sites which react with a derivatised epitope. Examples of suitable carrier proteins include bovine serum albumin (BSA), ovalbumin and keyhole limpet haemocyanin (KLH).

"Conjugate" refers to a molecule which is capable of linking an epitope to a carrier protein in a chemically non specific manner.

"Linker" refers to a molecule which is capable of undergoing a specific chemical reaction with both a carrier protein and an epitope so as to link the two together.

"Construct" refers to a carrier protein linked to a plurality of epitopes *via* linkers or conjugates.

A "charge balanced linker" is a linker which is charged such that when it reacts with a carrier protein, the overall surface charge pattern of the carrier protein remains essentially unchanged.

A "positive charge balanced linker" is a charge balanced linker carrying a positive charge.

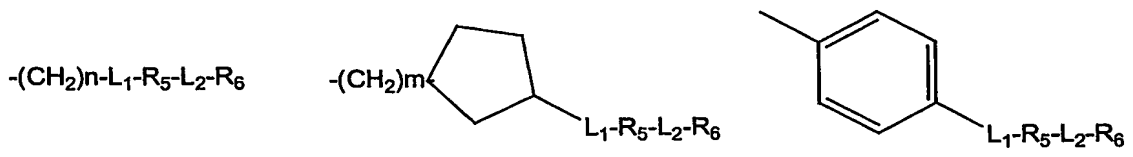
In the compounds of general formula (I), it is preferred that, independently or together:

X and Y are each independently oxygen;

R_1 is hydrogen, methyl or ethyl, with hydrogen being particularly suitable; and

R_2 is hydrogen or C_{1-4} alkyl with more preferred compounds having R_2 as hydrogen, methyl or ethyl, particularly hydrogen or methyl.

Within the definition of R_3, the positive nitrogen atom in R_5 needs to be an appropriate distance from the aromatic ring such that it does not adversely interfere with the aromatic ring electronics and hence ability to resonance stabilise carbocation **(12)**. Additionally, in order to facilitate synthesis from readily available starting reagents and incorporation of R_5, preferred R_3 substituents in general formula (II) are chosen from simple (*i.e.* unsubstituted) straight chain alkyl groups or simple cycloalkyl groups or simple aromatics containing a carboxylic acid. Particularly suitable cycloalkyl groups in R_3 are those which include a cyclopentyl or cyclohexyl moiety, while examples of aromatic groups include phenyl, alkyl phenyl (for example benzyl) or phenyl alkyl. Specific examples of suitable R_3 groups are:



wherein n = 2-6;

m = 1-3.

A preferred definition of R_5 provides a positive nitrogen atom that resembles as closely as possible the properties of the protein surface lysine residue that it is designed to mimic. Additionally, in order to facilitate the incorporation of R_5 within the framework detailed in general formula (II) from readily available starting reagents, it is preferred that the substituents NHR_5CO (where the NH is part of the L_1 moiety and the CO is part of the L_2 moiety) are chosen from simple amino acid residues that contain a side-chain protonatable amine functionality.

Also preferred in the definition of NHR_5CO is an amino acid residue that, through linker **(8)**, directly incorporates the charge-balance to the carrier protein. Thus, a high loading and soluble positive charge-balanced linker-carrier protein **(9)** results, which otherwise through the addition of a protected amine functionality in R_5 would provide an intermediate construct **(9)** containing a latent amine functionality and suffer from the previously described low solubility problems.

Suitable amino acid residues for NHR_5CO may be represented by the formula:

$$-NH-C[(CH_2)_pN^+R_8R_9R_{10}]CO-$$

wherein p is 1 to 3 and R_8, R_9 and R_{10} are as defined above.

For ease of synthesis and in order to avoid steric hindrance, the more suitable R_8, R_9 and R_{10} groups include hydrogen or C_{1-4} alkyl, with hydrogen and methyl being particularly preferred.

Within the definition of R_3, the substituent R_6 is defined as a spacer and is required to enable the smooth activation of linker **(8)** prior to formation of the *positive charge-balanced linker-carrier protein* **(9)**. It is well known in the art of peptide chemistry

that the activation of a non-urethane protected amino acid can lead to racemisation of the Cα-chiral centre (e.g. see Benoiton, N. L. and Kuroda, K. *Int. J. Pept. Prot. Res.* **17**, 197, 1981). Also, it is well known in the art of peptide chemistry that the activation of the non side-chain protected amino acids which are preferred for R_5 requires special conditions and often result in unwanted side reactions. Taking these considerations into account, the spacer R_6 is required to alleviate the above potential difficulties and provide an easily activated carboxylic acid functionality.

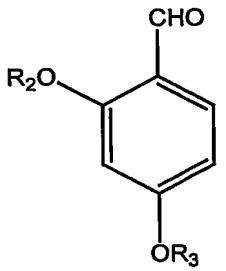
It is preferred that R_6 combines with an NH group derived from the L_2 moiety and the terminal COOH to form an amino acid residue of the formula:

$$-NH-(CH_2)_q-Z_s-(CH_2)_rCOOH;$$

where q and r are each 0 to 3, provided that both q and r are not both 0;
s is 0 or 1; and
Z is a 5-10 membered stable monocyclic or bicyclic aromatic ring or a 3-6 membered carbocyclic or alicyclic ring.

It is more preferred that r and s are 0 and q is 1 or 2.

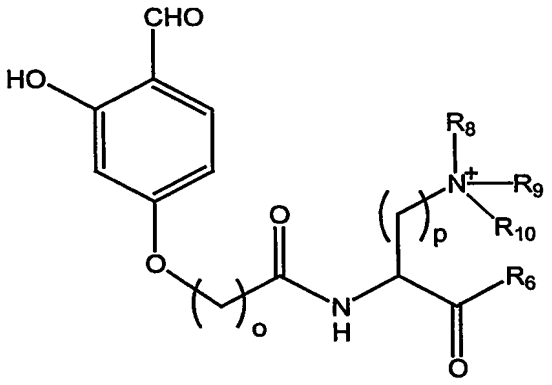
To facilitate synthesis of linker **(8)**, routes commencing from readily available starting reagents are preferred. Thus compounds of general formulae (Ia) are preferred, particularly linkers designed around a 2,4-dialkoxy substituted benzaldehyde as defined in general formula (II):



General formula (II),

which is a compound of General formula (Ia) in which X and Y are O, R_1 is H and R_2 and R_3 are as defined above.

A more preferred embodiment of the compound of General Formula II is detailed by general formula (III):



General formula (III)

wherein:

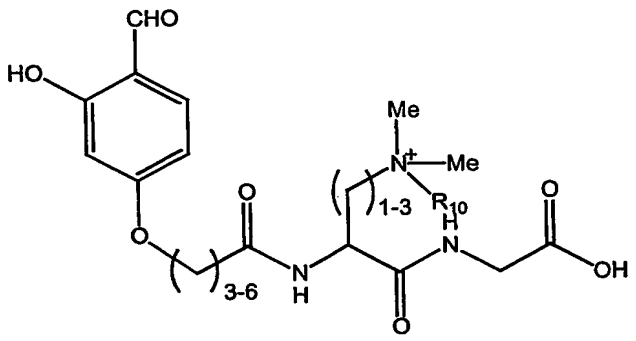
o is an integer from 2-6;

p is an integer from 1 to 3; and

R_6, R_8, R_9 and R_{10} are as defined above.

In the embodiment of General Formula (III), the combination NH-R_5CO (where NH forms part of the L_1 moiety and CO forms part of the L_2 moiety) is represented by an amino acid residue which contains a side chain with a quaternary nitrogen atom. The NH-R_5CO group can therefore replace the charge of a side chain lysine on a carrier protein which reacts with the carboxylic acid group attached to R_6.

A still more preferred embodiment of the compound of general formula (I) is detailed in general formula (IV):



General formula (IV)

Wherein R_{10} = H or Me.

Compounds of general formula (I) in which L_1 and L_2 are CONH can be synthesised by reacting a compound of general formula V:

$$H_2N\text{-}R_6\text{-}COOH \qquad (V)$$

wherein R_6 is as defined for general formula (I); and

wherein the compound of general formula (V) is bound at its C-terminus to a solid support;

with a compound of general formula (VI):

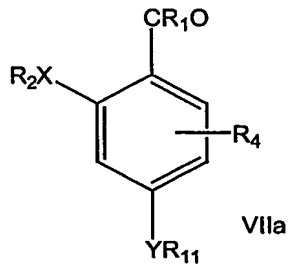
$$W\text{-}NH\text{-}R_5\text{-}COOH \qquad (VI)$$

wherein:

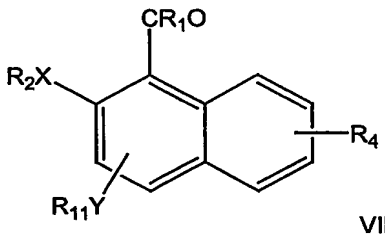
R_5 is as defined for general formula (I); and

W is a protecting group; and

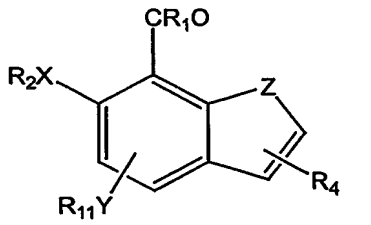
followed by removal of the protecting group and reaction with a compound of general formula (VII), which may be suitably protected as necessary:



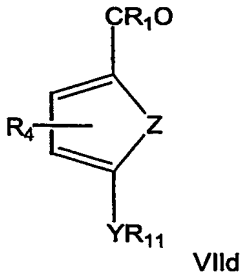
VIIa



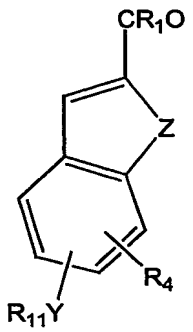
VIIb



VIIc



VIId



VIIe

General formulae (VII)

wherein

X, Y, Z, R_1, R_2 and R_4 are as defined for general formula I; and

R_{11} is C_{1-7} alkyl-COOH, C_{3-10} cycloalkyl-COOH or Ar-C_{0-7} alkyl-COOH

and subsequent removal of the product from the solid support.

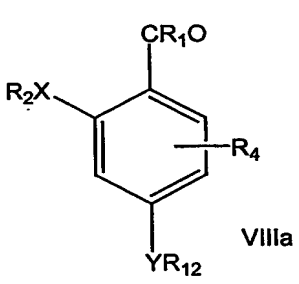
Suitable solid supports for use in the method include resins such as chlorotrityl resin. Removal from chlorotrityl resin can be achieved by treating the product with an acid, for example trifluoroacetic acid in a polar organic solvent such as dichloromethane.

The protecting group W may be a moiety such as Fmoc, which can be removed when required by treatment with piperidine in dimethylformamide.

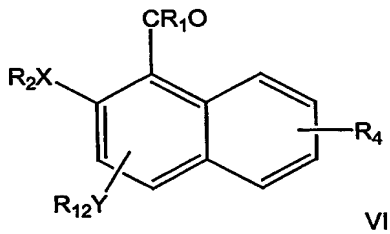
Compounds of general formulae (V), (VI) and (VII) are readily available and are well known to those of skill in the art.

As discussed in detail above, compounds of general formula (I) are of use for linking epitopes to carrier proteins.

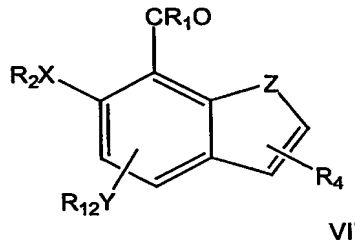
Therefore, in a further aspect of the invention, there is provided a compound of general formula (VIII):



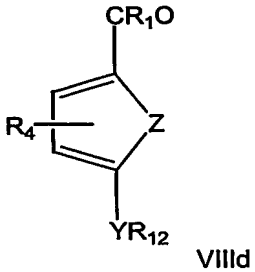
VIIIa



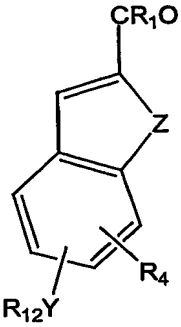
VIIIb



VIIIc



VIIId



VIIIe

General formulae (VIII)

wherein

X, Y, Z, R_1, R_2 and R_4 are as defined for general formula I; and

R_{12} is C_{1-7} alkyl-L_1-R_5-L_2-R_6CONHQ, C_{3-10} cycloalkyl-L_1-R_5-L_2-R_6CONHQ or Ar-C_{0-7} alkyl-L_1-R_5-L_2-R_6CONH-Q;

wherein

L_1, L_2 R_5 and R_6 are as defined in general formula (I); and

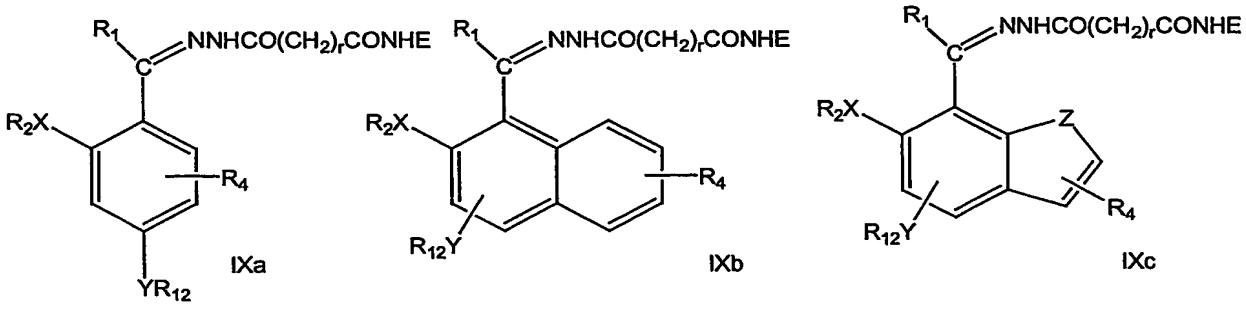
Q is the residue of a carrier protein having a lysine side chain from which the "NH" moiety in R_{12} is derived.

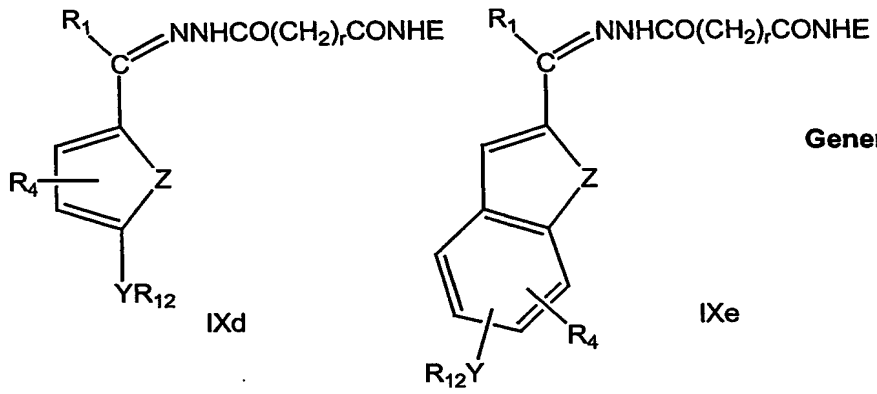
Suitable carrier proteins include bovine serum albumen, keyhole limpet haemocyanin, ovalbumin

The invention further comprises a process for the preparation of a compound of general formula (VIII) as defined above, the process comprising reacting a compound of general formula (I) as defined above with a carrier protein.

The reaction can be achieved by reacting a solution or suspension of the protein in an aqueous solvent with a compound of formula (I) or a derivative thereof, for example the succinimide ester in a solvent such as dimethyl sulfoxide at a temperature of from 15 to 50°C, but preferably at room temperature.

Compounds of general formula (VIII) are intended for linkage to a derivatised epitope and therefore, in a further aspect of the invention, there is provided a compound of general formula (IX):





General formulae (IX)

wherein X, Y, Z, R_1, R_2 and R_4 are as defined for general formula I;

R_{12} is as defined in general formula (VIII);

E is derived from an epitope; and

r is an integer from 1 to 5.

The epitope may be a fragment, for example an antigenic determinant, derived from a protein or peptide molecule or a variant or analogue of such a molecule. Examples of epitopes which can be used with this method include oxytocin and analogues thereof.

The compounds of general formula (IX) are simple to prepare from compounds of general formula (VIII) and thus, in a further aspect of the invention, there is provided a process for the preparation of a compound of general formula (IX) as defined above, the process comprising reacting a compound of general formula (VIII) as defined above with a compound of general formula (X):

$$E\text{-}NH\text{-}CO\text{-}(CH_2)_rCONHNH_2 \qquad (X)$$

where E and r are as defined above.

The reaction may be carried out in a hydrophilic organic solvent at a temperature of from 15 to 50°C but preferably at room temperature.

Compounds of general formula (X) can be prepared from an epitope by reacting a side chain of an epitope lysine residue with a compound of the formula:

$$HOOC-(CH_2)_rCONHNH_2$$

or a derivative thereof, such as a succinimide or maleimide derivative.

As already mentioned, compounds of general formula (IX) are of use in medical applications and therefore the invention further provides a compound of general formula (IX) for use in medicine.

One of the main medical applications is the use of a compound of general formula (IX) in a method for raising specific antibodies against the epitope E, the method comprising immunising a subject with a compound of general formula (IX).

Thus, the invention also provides a compound of general formula (IX) for immunising a subject in order to raise antibodies to the epitope E and the use of a compound of general formula (IX) in the preparation of an agent for raising antibodies against the epitope E.

Immunogenic compounds of general formula (IX) are of use as vaccines and therefore, in a further aspect of the invention there is provided a compound of general formula (IX) for use as a vaccine and also a pharmaceutical composition comprising a compound of general formula (IX) together with a pharmaceutically acceptable excipient.

The pharmaceutical composition may be a vaccine composition, in which case it may comprise a pharmaceutically acceptable adjuvant.

Other uses for the compounds of general formula (IX) are discussed below.

The technology described herein with particular exemplification in the controlled conjugation of *epitopes* to *carrier proteins*, has many additional potential applications, both in solution and on a solid phase support. Examples of such applications include but are not restricted to;

Solution phase applications

The chemical linkage of a carrier (examples of which include, but are not restricted to, peptides, proteins, sugars, lipids, nucleic acids, alkaloids, vitamins, etc.) to a ligand (examples of which include, but are not restricted to, peptides, proteins, sugars, lipids, nucleic acids, alkaloids, vitamins, etc.) using the composition of this invention. Solution phase applications of this invention include, but are not restricted to:

- Conjugation of epitopes/mimotopes to carrier proteins. The present invention enables higher epitope/mimotope concentrations to be loaded onto the carrier proteins with the retention of epitope/mimotope-carrier conjugate solubility, thus improving the immune response. Since the conjugation is a controlled process, more than one agent may be conjugated to the carrier protein, allowing carriage of single and multiple immunologically relevant epitopes/mimotopes (*e.g.* B-cell and T-cell epitopes/mimotopes). Conjugation of epitopes/mimotopes may also be combined with co-conjugation of immunomodulating compounds (*e.g.* lipids, adjuvants, immunostimulating DNA sequences, cytokines, *etc.*).

- Solution phase biochemical/biophysical/biomedical applications. Chemical linkage of a ligand to a carrier to enable molecular interactions to be monitored. In this case

the definition of ligands could be extended to include, but are not restricted to, chromophores (biochemical, biophysical or chemical), fluorophores (biochemical, biophysical or chemical), luminophores (biochemical, biophysical or chemical), phosphorescence, radiochemicals, quantum dots, electron spin tags, magnetic particles, nuclear magnetic resonance tags, x-ray tags, microwave tags, electrochemical, eletrophysical (*e.g.* increased resistance), surface plasmon resonance, calorimetry, etc . Using the present invention carriers would be tagged by a ligand, creating a soluble intermediate with which molecular interactions could be monitored by a complementary physical, chemical or biological technique.

Solid Phase Applications

The chemical linkage of a solid phase (*i.e.* non-solution phase) (examples of which include, but are not restricted to, synthetic materials (such as hydrocarbon-based plastics, polymers, glass, gels, resins, etc.), natural polymers such as proteins, sugars (*e.g.* cotton), lipids (liposomes), etc.) to a ligand (examples of which include, but are not restricted to, peptides, proteins, sugars, lipids, nucleic acids, alkaloids, vitamins, etc.) using the composition of this invention. Solid phase applications of the present invention include, but are not restricted to:

- Solid phase biochemical/biophysical applications. Chemical linkage of a ligand and/or carrier to a solid phase to enable molecular interactions to be monitored. In this case the present invention could be used for applications such as enzyme linked immunosorbent assays (ELISAs), surface plasmon resonance, *etc.* Selective covalent linkage of material to solid surfaces, will also allow generation of microarrays (including but not limited to peptides, proteins and nucleic acids).

- Separation/purification. Chemical linkage of a ligand to a solid phase enabling selective separation of molecules based on physicochemical properties. Applications include, but are not restricted to, affinity purification, chiral separation, *etc.*

- Medical Devices. Chemical derivatisation of medical devices and consumables allowing presentation of biologically active or inert molecules at a tissue/solid-surface interface. For example controlled conjugation of peptide growth factors, chemo-attractant proteins or analogues of both, to functionalised polymers commonly used in modern coverings, may allow development of next generation, bioactive wound dressings. In a further example, dialysis tubing may be derivatised with the linker in order to allow heparin to be coupled onto the surface of the polymer, decreasing the risk of contact activation of the blood coagulation process.

The invention will now be discussed in greater detail with reference to the drawings and the Examples, which are not intended to be limiting.

In the drawings:

FIGURE 1 shows the stoichiometric titration of BSA against the amine specific fluorescent reagent FLURAM 1™. By keeping one reactant constant and gradually increasing the other, a plateau was reached, indicating a poit of equivalence. Since the number of free amines in BSA is known, an estimate of the number taking part in the reaction was made (21-25).

FIGURE 2 is an electrophoresis gel showing the molecular weight of BSA compared with that of three BSA constructs, TML85, Tfa85 and BAL85.

FIGURE 3 is plot of AU at 550nm vs. log concentration and illustrates the solubility of linker BSA constructs (20-22) in 10nM ammonium bicarbonate at pH 8.

FIGURE 4 is a plot of AU at 550nm vs. log concentration and illustrates the solubility of linker-BSA constructs (20-22) in 0.1M sodium formate at pH 4.5.

FIGURE 5 is a plot of AU at 550nm vs. log concentration and illustrates the solubility of linker-BSA constructs (20-22) in 10 mM potassium phosphate at pH 6.

FIGURE 6 is a plot of AU at 550nm vs. log concentration and illustrates the solubility of linker-BSA constructs (24 and 27) in 10 mM potassium phosphate at pH 7.

FIGURE 7 is an electrophoresis gel showing the molecular weight of BSA compared with that of BSA.BAL, BAL55-Conj, BSA.TML and TML-conj.

FIGURE 8 shows the HPLC analysis of the BSA-TML85-epitope conjugate (24) after hydrolysis with 1N hydrochloric acid and shows that hydrolysis regenerated BSA-TML85 and the epitope (13).

FIGURES 9 TO 11 show the results of ELISA analyis of sera from mice immunised with BSA alone (Figure 9) or with oxytocin conjugated to BSA using either BAL linker (Figure 10) or TML linker (Figure 11). Both constructs are recognised by antibodies raised to BSA alone, which is to be expected since BSA is the carrier protein present within both constructs and thus provides a positive control.

FIGURE 9 shows that titres of antibodies that recognise both BSA-BAL55-oxytocin and BSA-TML-oxytocin constructs are raised in mice immunised with BSA alone.

FIGURE 10 shows that titres of BSA (non-specific) and BSA-BAL55-oxytocin construct (specific) are raised in mice immunised with BSA-BAL55-oxytocin construct.

FIGURE 11 shows that titres of BSA (non-specific) and BSA-TML85-oxytocin construct (specific) antibodies are raised in mice immunised with BSA-TML85-oxytocin construct.

The experiments described below exemplify the utilisation of a *charge-balanced linker* in the controlled conjugation of an *epitope* to bovine serum albumin (BSA) carrier protein. A series of *in vitro* solubility and *in vivo* immunisation experiments is described that clearly shows the superior characteristics of a charge-balanced linker construct for the generation of an antibody response to an immunogen. The exemplification is described as follows;

1. Synthesis of example epitope and linker structures
2. Solubility studies with BSA-linker constructs
3. Solubility studies with BSA-linker-epitope constructs
4. Chemical analysis of BSA-linker-epitope constructs
5. Immunisation studies with BSA-linker-epitope constructs

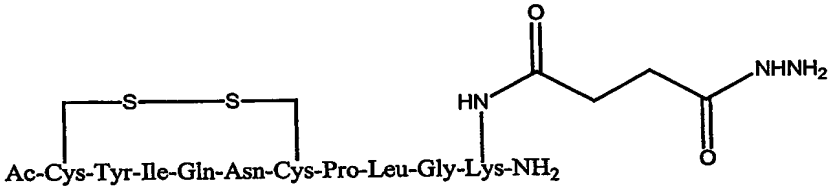
Experimental Methods

All reagents were of the highest commercially available quality and were used as received. Unless otherwise stated all chemicals and biochemicals were purchased from the Sigma Chemical Company (Poole, Dorset, UK). All solid phase synthesis was performed using an "Fmoc/tBu" procedure, (see Atherton, E and Sheppard, R. C. in 'Solid Phase Peptide Synthesis: A Practical Approach', IRL Press, **1989**. Standard Fmoc amino acids were obtained from Chem-Impex International (Wood Dale, IL, USA) and Novabiochem (Nottingham, UK) with the exception of Fmoc N-ε-trimethyllysine, which was purchased from Bachem UK Ltd. (St. Helens, UK), along with Fluram (fluorescamine). PS-carbodiimide resin was obtained from Argonaut Technologies (Muttenz, Switzerland). All solvents were purchased from Romil (Cambridge, UK). Solid phase syntheses were performed manually in a polypropylene syringe fitted with a polypropylene frit to allow filtration under vacuum. Analytical HPLC was performed on Agilent 1100 series instruments including a G1311A quaternary pumping system, with a G1322A degassing module and a G1365B multiple wavelength UV-VIS detector. Data were collected and integrated with Chemstation

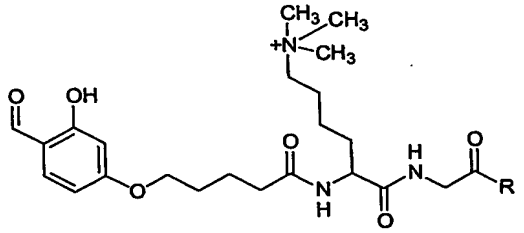
2D software. The analyses were performed on a Zorbax, 5μm, C8 reverse phase column (150 x 4.6 mm i.d.), at a flow rate of 1.5 ml/min, monitoring at 215 and 254 nm. Eluents used were (A) 0.1% trifluoroacetic acid in water and (B) 90% acetonitrile/10% eluent A and used to run a gradient starting at 10% B, increasing to 90% B over 7 minutes, holding for 1 minute, returning to 10% B over 1 minute and then remaining at initial conditions for a further 4 minutes to allow column re-equilibration. Compounds were purified by semi-preparative HPLC, using a Phenomenex Jupiter C4 reverse phase column (250 x 10 mm i.d.) at a flow rate of 4 ml/min, using the equipment and eluents described above. The molecular weight of compounds was determined on an Agilent 1100 series LC/MSD electrospray mass spectrometer. BSA conjugates were concentrated using Centricon centrifugal filters (50,000 MWCO) (Millipore, MA, USA) and purified by dialysis using Slide-A-Lyser dialysis cassettes (10,000 MWCO) (Pierce, IL, USA). Molecular weight estimations and purity of the BSA conjugates were made polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) using 4-20% NuPAGE gels (Invitrogen, Paisley, U.K.) employing the 3-(N-morpholino) propane sulfonic acid (MOPS) buffer system (Invitrogen) according the manufacturers instructions. Protein visualisation was carried out using the SilverExpress stain kit (Invitrogen). In all cases the gels were dried using the gel drying kit (Invitrogen) and for presentation purposes, gels were scanned at 300 dpi resolution using grey scale false colour (OfficeJet Pro1175c; Hewlett Packard). Fluram fluorescence assays were carried out in Microfluor W1 96-well microtitre plates (Dynex Thermo Lifesciences, UK) using a Gemini plate reader (Molecular Devices, Crawley, UK) and monitored at 390 nm (excitation) and 460 nm (emission). Turbidity measurements were made at 650 nm using a Spectramax384 96-well plate reader (Molecular Devices), carried out in 384-well PS microplates (Labsystems, Basingstoke, Hants, UK), while Bradford assays were measured at 595 nm in 96-well PS microplates (Greiner Bio-One Ltd., Stonehouse, Gloucestershire, UK).

Example 1 - Synthesis of Example Epitope and Linker Structures

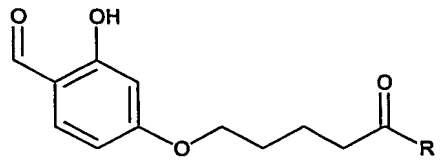
Synthesis of the oxytocin analogue **(13)** and linkers **(14-19)** proceeded smoothly using standard solution chemistries and Fmoc solid phase techniques (see Atherton, E and Sheppard, R. C. in 'Solid Phase Peptide Synthesis: A Practical Approach', IRL Press, **1989)** to provide the desired compounds in good yield and purity. The linkers were stored as the free acids **(14, 16, 18)** and the activated succinimide ester of the linkers **(15, 17, 19)** were freshly prepared when required.



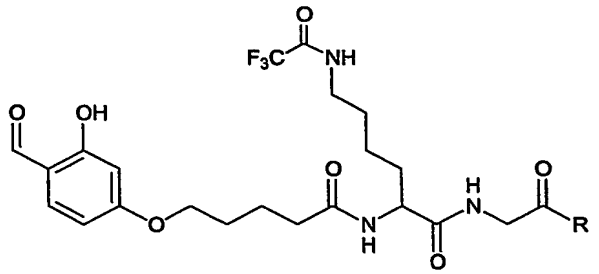
Oxytocin analogue **(13)**



Trimethyllysine linker (TML) **(14)** R = OH
(15) R = OSu



Backbone amide linker (BAL) **(16)** R = OH
(17) R = OSu



Trifluoroacetyllysine linker (Tfa) **(18)** R = OH
(19) R = OSu

A. Synthesis of Oxytocin Analogue (13)

Oxytocin analogue **(13)** Peptide epitope **(13)** with the sequence (one letter code) Acetyl-CYIQNCPLGK($COCH_2CH_2CONHNH_2$)-NH_2, was synthesised manually using Fmoc/tBu protection strategy on TGR resin (0.25 g, 0.05 mmol, substitution: 0.2 mmol/g). Coupling of the Fmoc amino acids was accomplished with an HBTU/HOBt method utilising dimethylformamide as the solvent, using 3 equivalents of amino acid and coupling reagents with respect to the loading of the resin. The Fmoc group was removed by a 15 min treatment with 20% piperidine in dimethylformamide. The C-terminal lysine residue was introduced with Dde side chain protection, to allow orthoganol deprotection at a later stage in the synthesis. After Fmoc deprotection of the final residue, the N-terminus was acetylated using acetic anhydride (48 µL, 0.5 mmol) and diisopropylethylamine (43 µL, 0.25 mmol) in dimethylformamide for 2 hours and the Dde protection of the lysine side chain removed with 2% hydrazine in dimethylformamide for 15 mins. The free amine of the lysine side chain was extended

by reaction with succinic anhydride (50 mg, 0.5 mmol) and diisopropylethylamine (43 μL, 0.25 mmol) in dimethylformamide for 2 hours and then hydrazine, coupled as a 10% solution in dimethylformamide using HBTU/HOBt (in excess) for 3 hours. Final cleavage of the peptide from the resin was performed with 92.5% trifluoroacetic acid / 2.5% triisopropylsilane / 2.5% water / 2.5% ethanedithiol (40 mL/g resin) for 75 mins. The resin was removed by filtration and the filtrate was concentrated by sparging with nitrogen. The crude product was precipitated and washed with cold methyl tert-butyl ether (3 x 50 mL), before being re-dissolved in 50% (aq.) acetonitrile and lyophilised. The peptide was re-dissolved in ammonium bicarbonate (0.1 M, pH 8) to a concentration of 100μM and oxidised using hydrogen peroxide (1.5 eq) for 45 mins. The reaction was monitored by LC-ESI-MS and with Ellman's reagent and finally quenched with 10% (aq) acetic acid (in excess). The mixture was lyophilised once more and then purified by semi-preparative RP-HPLC. Yield: 24mg, 0.019 mmol, 37%. ESI-MS *m/z*: 1291.3 (calc. for $M + H^+$ 1291.5). HPLC retention time: 3.44 mins.

B. Synthesis of {5*S*-(Carboxymethylcarbamoyl)-5-[5-(4-formyl-3-hydroxy-phenoxy)pentanoyl amino]pentyl}trimethylammonium **(14)**.

The compound was synthesised manually using Fmoc/tBu protection strategy on 2-chlorotrityl resin (0.19 g, 0.19 mmol), pre-loaded with glycine (substitution: 1.0 mmol/g). Fmoc-Lys$(Me)_3$-OH was double coupled using an HBTU/HOBt method with dimethylformamide as the solvent and 3 equivalents of amino acid and coupling reagents with respect to the loading of the resin. The Fmoc group was removed by a 15 min treatment with 20% piperidine in dimethylformamide. Coupling of 5-(4-formyl-3-hydroxyphenoxy) pentanoic acid (BAL) **(16)** was accomplished with a benzotriazole-1-yl-oxy-tris-(dimethylamino)phosphoniumhexafluoro phosphate (BOP) (BOP/HOBt) method utilising dimethylformamide as the solvent and 3 equivalents of **(16)** and coupling reagents with respect to the loading of the resin. A final 20% piperidine treatment was included to remove any ester formed at the 2-hydroxyl position of the BAL. Final cleavage of the linker from the resin was performed with several treatments of 5% trifluoroacetic acid in dichloromethane, each for 5 mins. The

resin was removed by filtration and the pooled filtrate was concentrated by sparging with nitrogen. The crude product was precipitated and washed with cold methyl tert-butyl ether, before being re-dissolved in 30% (aq) acetonitrile and lyophilised. Finally, the compound was purified by semi-preparative RP-HPLC, the pure fractions pooled and lyophilised once more to yield an off white solid. Yield: 35 mg, 0.075 mmol, 39%. ESI-MS *m/z*: 466.2 (calc. for M + H^+ 466.26). HPLC retention time: 3.75 mins.

C. {5*S*-[(2,5-Dioxopyrrolidin-1-yloxycarbonylmethyl)carbamoyl]-5-[5-(4-formyl-3-hydroxyphenoxy)pentanoylamino]pentyl}trimethylammonium **(15)**.

Compound **(14)** (35 mg, 0.075 mmol) was dissolved in dimethylformamide (2 mL) and added to a stirred solution of PS-carbodiimide (288 mg, 0.375 mmol) in dichloromethane (10 mL). The mixture was stirred for 20 mins before the addition of N-hydroxysuccinimide (9 mg, 0.075 mmol) dissolved in dimethylformamide (1 mL). The reaction was then stirred at room temperature and monitored by HPLC until completion (5 hours). The resin was removed by filtration, the solvent removed *in vacuo* and the compound used without further purification. Yield: 38 mg, 0.068 mmol, 90%. ESI-MS *m/z*: 563.3 (calc. for M + H^+ 563.3). HPLC retention time: 4.16 mins.

D. 5-(4-formyl-3-hydroxyphenoxy)pentanoic acid (BAL) **(16)**.

2,4-Dihydroxybenz aldehyde (10 g, 0.072 mol) and spray-dried potassium fluoride (8.4 g, 0.144 mol) were stirred vigorously at 60°C for 20 mins in anhydrous acetonitrile (150 mL). Methyl-5-bromovalerate (42.3 g, 0.216 mol) was added in one portion and the mixture brought to a gentle reflux for 5 hours. The reaction was allowed to cool to room temperature and the solvent removed *in vacuo*. The residue was partitioned between water (100 mL) and ethyl acetate (50 mL). The aqueous was washed twice more with ethyl acetate (2 x 30 mL) and the combined organic back-washed with water, dried over anhydrous magnesium sulphate, filtered and evaporated to dryness. The resulting red oil was re-crystallised from ether (30 mL) and heptane (20 mL). The methyl ester obtained was dissolved in tetrahydrofuran (120 mL) and stirred vigorously at room temperature. To this solution was added lithium hydroxide

(3.7 g, 0.088 mol) dissolved in water (60 mL) and the mixture stirred for 4 hours. The solvent was reduced *in vacuo* and the resultant oily residue diluted with water (30 mL), washed twice with methyl tert-butyl ether (2 x 50 mL), acidified to pH 2 with conc. Hydrochloric acid and extracted with ethyl acetate (4 x 30 mL). The combined ethyl acetate was dried over anhydrous magnesium sulphate, filtered and evaporated to dryness to give a white solid product. Yield: 9.86 g, 0.041 mol, 57%. ^{1}H NMR ($CDCl_3$) δ: 11.26 (2H, br.s), 9.69 (1H, s), 7.41 (1H, d, J=8.6 Hz), 6.51 (1H, dd, J=8.6, 2.2 Hz), 6.40 (1H, d, J=2.2 Hz), 4.02 (2H, t, J=5.9 Hz), 2.44 (2H, t, J=7.0 Hz), 1.84 (4H, m). mp: 88-91°C. ESI-MS *m/z*: 239.1 (calc. for M + H^+ 239.08). HPLC retention time: 5.34 mins.

E. 5-(4-formyl-3-hydroxyphenoxy)pentanoic acid 2,5-dioxopyrrolin-1-yl ester (BAL-OSu) **(17).**

PS-carbodiimide resin (4.2 g, 5.5 mmol) was suspended in dichloromethane (45 mL) stirred for 5 mins to swell the resin. Compound **(16)** (1.0 g, 4.2 mmol) was added, dissolved in dichloromethane (10 mL) and the resin mixture stirred for a further 20 mins before the addition of N-hydroxysuccinimide (0.46 g, 4.0 mmol) dissolved in dimethylformamide (4 mL). The reaction was then stirred at room temperature and monitored by HPLC until completion (18 hours). The resin was removed by filtration, the solvent removed *in vacuo* and the final product re-crystallised from isopropanol. Yield: 1.3 g, 3.8 mmol, 92%. ESI-MS *m/z*: 336.1 (calc. for M + H^+ 336.1). HPLC retention time: 6.18 mins.

F. [2S-[5-(4-formyl-3-hydroxyphenoxy)pentanoylamino]-6-(2,2,2-trifluoroacetyl amino)hexanoylamino]acetic acid (Tfa) **(18).**

The compound was synthesised manually by solid phase synthetic methods, using Fmoc/tBu protection strategy on 2-chlorotrityl resin (0.3 g, 0.3 mmol), pre-loaded with glycine (substitution: 1.0 mmol/g). Coupling of Fmoc-Lys(Tfa)-OH was accomplished with a 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate/N-

hydroxybenzotriazole (HBTU/HOBt) method utilising dimethylformamide as the solvent, using 3 equivalents of amino acid and coupling reagents with respect to the loading of the resin. The Fmoc group was removed by a 15 min treatment with 20% piperidine in dimethylformamide. Coupling of 5-(4-formyl-3-hydroxyphenoxy) pentanoic acid (BAL) **(16)** was achieved as above using BOP activation. A final 20% piperidine treatment was included to remove any ester formed at the 2-hydroxyl position of the BAL. Final cleavage of the linker from the resin was performed with several treatments of 5% trifluoroacetic acid in dichloromethane, each for 5 mins. The resin was removed by filtration and the pooled filtrate was concentrated by sparging with nitrogen. The crude product was precipitated and washed with cold methyl tert-butyl ether, before being re-dissolved in 50% (aq) acetonitrile and lyophilised. Finally, the compound was purified by semi-preparative RP-HPLC, the pure fractions pooled and lyophilised once more to yield a white solid. Yield: 49 mg, 0.095 mmol, 32%. ESI-MS *m/z*: 520.2 (calc. for M + H^+ 520.1). HPLC retention time: 5.12 mins.

G. [2S-[5-(4-formyl-3-hydroxyphenoxy)pentanoylamino]-6-(2,2,2-trifluoroacetyl amino)hexanoylamino]acetic acid 2,5-dioxopyrrolin-1-yl ester (Tfa-OSu) **(19).**

Compound **(18)** (49 mg, 0.095 mmol) was dissolved in dimethylformamide (2 mL) and added to a stirred solution of PS-carbodiimide (375 mg, 0.475 mmol) in dichloromethane (10 mL). The mixture was stirred for 20 mins before the addition of N-hydroxysuccinimide (11 mg, 0.095 mmol) dissolved in dimethylformamide (1 mL). The reaction was then stirred at room temperature and monitored by HPLC until completion (5 hours). The resin was removed by filtration, the solvent removed *in vacuo* and the compound used without further purification. Yield: 55 mg, 0.089 mmol, 93%. ESI-MS *m/z*: 617.2 (calc. for M + H^+ 617.1). HPLC retention time: 5.64 mins.

Example 2 – Solubility Studies with BSA-linker Constructs

Fluram Assay.

Test sample or standard (10 µL) was added to an assay plate well containing di-basic sodium hydrogen phosphate buffer (85 µL). Fluram was dissolved in acetonitrile (1

mg/mL) and 5 μL of this solution was added to each well, mixed and allowed to react for 5 mins before a fluorescence reading was obtained.

Stoichiometric Evaluation of BSA Acylation.

BSA was dissolved in 0.1 M sodium acetate (pH 7.25) to produce a 10 mg/mL solution, of which 10 μL was transferred (in triplicate) into wells containing 160 μL di-basic sodium hydrogen phosphate buffer (0.1 M, pH 8.2). 85 μL of the samples was transferred across the plate with double dilution into di-basic sodium hydrogen phosphate buffer. Fluram was dissolved in acetonitrile (20 μg/mL) and 5 μL (0.1 μg, 359 pmol) of this solution was added to each well, mixed and allowed to react for 5 mins before a fluorescence reading was obtained.

Preparation of BSA-Linker Constructs **(20,21,22).**

BSA (2 mg, 29 nmol) was dissolved in 0.1 M sodium acetate (1 mL, pH 7.25) and added to BAL-OSu **(17)** (2.5 mg, 7.46 μmol), Tfa-OSu **(19)** (4.6 mg, 7.46 μmol) or TML-OSu **(15)** (4.2 mg, 7.46 μmol) each dissolved in dimethyl sulfoxide (0.5 mL). The reactions were stirred at room temperature and the disappearance of free amine monitored with Fluram. Once complete (approx. 2-3 hours), the reaction mixtures were dialysed (3 x 2 L) against 10 mM ammonium bicarbonate (pH 8) and the products analysed by gel electrophoresis.

Bradford Assay.

A standard BSA solution (0.5 mg/mL) was prepared and a range of volumes (0 – 15 μL) added to wells containing water to give a total volume of 100 μL. In a similar manner, 5 μL of the test sample was added to wells (in triplicate) containing water (95 μL). Bradford reagent (100 μL) was then added to both standard and test wells and the solutions mixed with a multi-channel pipette. The plate was then left at room temperature for 5 mins before UV measurements were taken. Protein concentrations were determined by comparison with the standard curve generated for BSA.

Solubility Measurements of BSA-Linker Constructs **(20,21,22)** at pH 8.

1 mL of the BSA-linker constructs **(20,21,22)** in ammonium bicarbonate buffer was concentrated to approximately a fifth of its original volume by centrifugal filtration and the protein concentration assessed by a Bradford assay. The concentration of the BSA-linker constructs **(20)** and **(22)** was adjusted to 5 mg/mL while the preparation derived from **(21)** was adjusted to 4 mg/mL. 40 μL of each solution was transferred into wells (in triplicate) of a 384-well microtitre plate and 20 μL of each sample was transferred across the plate with double dilution into ammonium bicarbonate buffer. The samples were allowed to come to equilibrium over 30 mins before turbidity measurements were taken.

Solubility Measurements of BSA-Linker Constructs **(20,21,22)** at pH 4.5.

1 mL volumes of the BSA-linker constructs **(20,21,22)** in ammonium bicarbonate buffer were concentrated to approximately a fifth of their original volume by centrifugal filtration. These filters were then employed in solvent exchange process to replace the original ammonium bicarbonate buffer with a sodium formate buffer (0.1 M, pH 4.5). This was achieved through cycles of dilution and concentration with the new buffer (approx. 5-6 cycles) until the theoretical ammonium bicarbonate content was below 1%. The protein content of the concentrated preparations (now in formate buffer) was then assessed by a Bradford assay. 40 μL of each solution was transferred into wells (in triplicate) of a 384-well microtitre plate and 20 μL of each sample was transferred across the plate with double dilution into sodium formate buffer (0.1 M, pH 4.5). The samples were allowed to come to equilibrium over 30 mins before turbidity measurements were taken.

Results and Discussion

Solubility of BSA-Linker Constructs **(20-22).** In order to assess the approximate number of free amines in bovine serum albumin (BSA) carrier protein, that were available for conjugation, a stoichiometric evaluation of the reaction between an amine specific fluorescent reagent (Fluram) and BSA was performed. By keeping one

reactant constant and gradually increasing the other, a plateau was reached indicating a point of equivalence (Figure 1). Since the number of free amines in BSA is known an estimate of the number taking part in the reaction was made (approx. 21-25).

Three BSA-linker constructs containing TML85 **(20)**, Tfa85 **(21)** and BAL85 **(22)** were initially prepared, via coupling with activated linkers **(15, 17, 19)**, to approximately 85% - 90% loading of accessible surface amines (estimated by Fluram monitoring). Characterisation of the BSA modified constructs by gel electrophoresis confirmed the expected increase in molecular weight compared with the native BSA (Figure 2).

The BSA-TML85 **(20)** construct proved a highly modified protein that retained good aqueous solubility over a wide pH range, whereas BSA constructs derived from the BAL and Tfa linkers were less soluble. At pH 8, BSA-TML85 **(20)** and BSA-BAL85 **(22)** showed reasonably good solubility at around 2-3 mg/mL, while BSA-Tfa85 **(21)** precipitated around 0.5 mg/mL (Figure 3).

At more acidic conditions (pH 4.5) BSA-BAL85 **(22)** and BSA-Tfa85 **(21)** exhibited low solubility and precipitated at concentrations above 250 µg/mL, whereas BSA-TML85 **(20)** possessed solubility well above 3.5 mg/mL (Figure 4). This is a particularly important finding since, as detailed earlier, the conjugation reaction between construct and epitope is chemoselective at acidic pH, (ideally performed at pH 4 - 4.5). Thus poor solubility of BSA-linker constructs at acidic pH is extremely detrimental in the formation of highly loaded BSA conjugates.

EXAMPLE 3 – Solubility Studies with BSA-linker-epitope Constructs

Preparation of BSA-Linker Construct **(23).**

BSA (2 mg, 29 nmol) was dissolved in 0.1 M sodium acetate (1 mL, pH 7.25) and added to BAL-OSu **(17)** (0.25 mg, 0.746 µmol) dissolved in dimethyl sulfoxide (0.5 mL). The reaction was stirred at room temperature and the disappearance of free

amine monitored with Fluram until approx. 55% acylation had been achieved (approx. 2 hours). The reaction mixture was dialysed (3 x 2 L) against 10 mM ammonium bicarbonate (pH 8) and the products analysed by gel electrophoresis.

Hydrazone Conjugation of Epitope **(13)** to BSA-Linker Constructs **(20,23)** Providing Conjugates **(24,27)**

2 mL of BSA-linker constructs **(20)** and **(23)** in ammonium bicarbonate buffer were dialysed (3 x 4 L) against sodium formate buffer (0.1 M, pH 4) producing a final concentration of approx. 1 mg/mL. Oxytocin analogue **(13)** (2.6 mg, 2.1 μmol) was dissolved in dimethyl sulfoxide (1.6 mL) and added to the BSA-linker construct solutions (2 mL), the final content of dimethyl sulfoxide being approximately 45%. The solutions were stirred at room temperature for 18 hours and dialysed (3 x 2 L) against 10 mM PBS (pH 7.4). The conjugates **(24)** and **(27)** were characterised by gel electrophoresis.

Solubility Measurements of BSA Conjugates **(24,27)**.

1 mL volumes of the BSA conjugates **(24,27)** in 10 mM phosphate buffer, at the chosen pH, were concentrated to approximately a fifth of their original volume by centrifugal filtration and the protein content of the concentrated preparations was then measured by a Bradford assay. 40 μL of each solution was transferred into wells (in triplicate) of a 384-well microtitre plate and 20 μL of each sample was transferred across the plate with double dilution into the phosphate buffer. The samples were allowed to come to equilibrium over 30 mins before turbidity measurements were taken.

Results and Discussion

Preparation and Solubility of BSA-linker-epitope Conjugates **(24-27)**.

The neurohypophysial hormone oxytocin, is a disulfide constrained nonapeptide (cyclo-[CYIQNC]PLG), and was chosen as a model epitope with which to carry out conjugation and immunisation studies. Typically, the conjugation reactions between

BSA-linker constructs **(20-22)** and epitope **(13)** were performed in an aqueous buffer/dimethyl sulfoxide medium at pH4 - 4.5. Loading reactions were complete after approximately 18 hours, using 2-3 equivalents of the oxytocin analogue hydrazide **(13)** with respect to the number of moles of aldehyde accessible for conjugation. Initially, only conjugates BSA-TML85-epitope13 **(24)** and BSA-BAL85-epitope13 **(26)** were adequately prepared since the poor solubility of the BSA-Tfa85 **(21)** construct hampered any synthetic efforts to produce the conjugate BSA-Tfa85-epitope13 **(25)**. Upon dialysis into phosphate buffer (pH 7.4), however, the conjugate obtained from BSA-BAL85-epitope13 **(26)**, precipitated and aggregated becoming very poorly soluble. In contrast, the conjugate BSA-TML85-epitope13 **(24)** remained relatively soluble with only a slight precipitate seen in the solution. In order to proceed with immunisation studies, a soluble conjugate based around BAL linker **(17)** was required and such a conjugate, BSA-BAL55-epitope13 **(27)**, was obtained with a reduced surface loading of approximately 55%, through BSA-BAL55 **(23)**. Solubility studies showed that the BSA-TML85-epitope13 **(24)** and BSA-BAL55-epitope **(27)** conjugates had good solubility at pH 6 and pH 7.4 of around 0.5 – 1 mg/mL (Figures 5 and 6).

Gel electrophoresis confirmed the increase in molecular weight compared with the native BSA (Figure 7).

EXAMPLE 4 – Chemical Analysis of BSA-linker-epitope Constructs

Hydrolysis of BSA-TML-Oxytocin Conjugate **(24)**.

Equal volumes of conjugate and 1N hydrochloric acid were mixed and assayed by LC-MS every 15 mins.

Results and Discussion

Hydrolysis of BSA-TML85-epitope13 conjugate **(24)**. Reversibility of the linkage between carrier protein and epitope is fundamentally crucial to quality control of the conjugate production process. Unless the chemical integrity of the loaded epitope can

be confirmed post-conjugation, the validity of any results obtained with the conjugate must be treated with caution.

Acid hydrolysis of the hydrazone bond within BSA-TML85-epitope13 conjugate **(24)** regenerated BSA-TML85 **(20)** and epitope **(13)**. The reaction progressed smoothly over 1hr, with clear identification of epitope **(13)** by LC-ESI-MS (Figure 8). Analysis for a free thiol within this hydrolysis product with Ellman's reagent proved negative, confirming the integrity of the disulfide bond within epitope **(13)**.

EXAMPLE 5 – Immunisation Studies

Mice were immunised with BSA alone or with oxytocin conjugated to BSA using either BAL linker or TML linker.

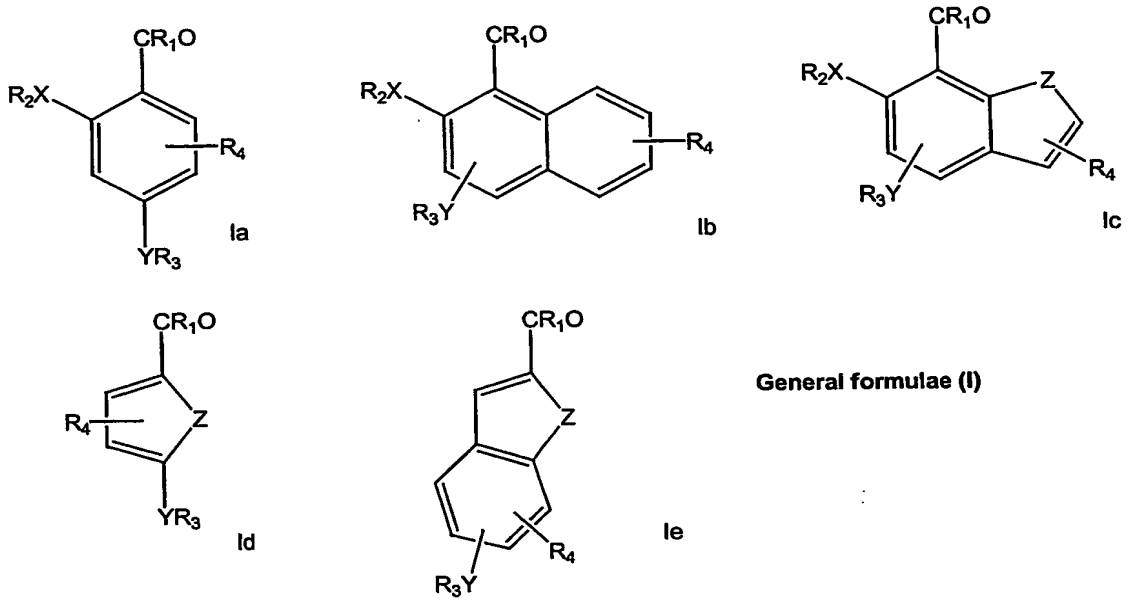
The results are shown in Figures 9-11. Figure 9 shows that both constructs are recognised by antibodies raised to BSA alone, which is to be expected since BSA is the carrier protein present within both constructs, and thus provides a positive control.

Comparison of the antibody titres raised against the BSA-BAL55-oxytocin construct and the BSA-TML85-oxytocin construct reveals distinct differences in the nature of the antibodies produced. Results from mice immunised with the BSA-BAL55-oxytocin construct (Figure 10) show a greater proportion of BSA (non-specific) antibodies produced than those antibodies specific for the whole construct itself. In contrast, mice immunised with the BSA-TML85-oxytocin construct (Figure 11) show the converse; the proportion of specific antibodies raised to the whole construct is greater than the non-specific BSA titres.

These results seem to indicate that the TML85 construct has greater epitope surface coverage and greater aqueous solubility than the BAL55 construct. This is supported by the results of Examples 1 to 4 described above.

CLAIMS

1. A positive charge-balanced linker according to general formulae (Ia to Ie):



General formulae (I)

wherein:

X = O or S;

Y is O, S or CHR_4;

Z is O or S;

R_1 is H or C_{1-7} alkyl;

R_2 is H or C_{1-7} alkyl;

R_3 is C_{1-7} alkyl-L_1-R_5-L_2-R_6, C_{3-10} cycloalkyl-L_1-R_5-L_2-R_6 or Ar-C_{0-7} alkyl-L_1-R_5-L_2-R_6;

R_4 is H or C_{1-7} alkyl at any vacant position on the aromatic ring;

each of L_1 and L_2 is absent or a suitable linker such as CONH;

R_5 is C_{1-7} alkyl or C_{3-6}-cycloalkyl or Ar-C_{0-7}-alkyl containing a trisubstituted nitrogen atom $NR_7R_8R_9$, or is a quaternary nitrogen atom $N^+R_7R_8R_9R_{10}$ such that the substituent R_5 contains a positively charged nitrogen atom;

R_5 is C_{1-7} alkyl, C_{3-10} cycloalkyl or Ar-C_{0-7} alkyl each of which is substituted with a trisubstituted nitrogen atom $NR_7R_8R_9$ or a quaternary nitrogen atom $N^+R_7R_8R_9R_{10}$, such that R_5 contains a positive charge;

each of R_7, R_8, R_9 and R_{10} is independently hydrogen, C_{1-7} alkyl, C_{3-10} cycloalkyl or Ar-C_{0-7} alkyl, or any two or more of R_7, R_8, R_9 and R_{10} together form an alicyclic or arylalicyclic ring system;

R_6 is C_{1-7} alkyl-COOH, C_{3-10} cycloalkyl-COOH or Ar-C_{0-7} alkyl-COOH;

or a salt, hydrate, solvate, complex or prodrug thereof.

2. A compound as claimed in claim 1 wherein, independently or in any combination:

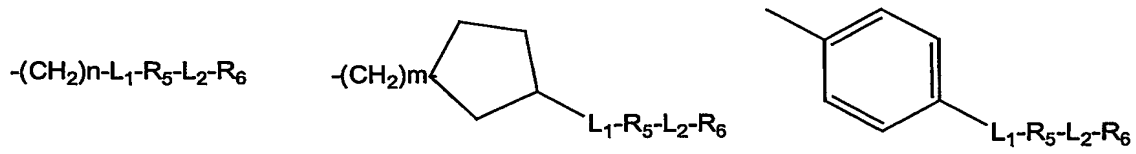
X and Y are each independently oxygen;

R_1 is hydrogen, methyl or ethyl, with hydrogen being particularly suitable; and

R_2 is hydrogen or C_{1-4} alkyl.

3. A compound as claimed in claim 2, wherein R_2 is hydrogen or methyl.

4. A compound as claimed in any one of claims 1 to 3 wherein R_3 comprises



wherein n = 2-6;
m = 1-3.

5. A compound as claimed in any one of claims 1 to 4, wherein NHR_5CO (where the NH is part of the L_1 moiety and the CO is part of the L_2 moiety) comprises a simple amino acid residues that contain a side-chain protonatable amine functionality.

6. A compound as claimed in claim 5, wherein NHR_5CO is represented by the formula:

$$-NH-C[(CH_2)_pN^+R_8R_9R_{10}]CO-$$

wherein p is 1 to 3 and R_8, R_9 and R_{10} are as defined above.

7. A compound as claimed in any one of claims 1 to 6 wherein R_8, R_9 and R_{10} groups are each independently hydrogen or C_{1-4} alkyl.

8. A compound as claimed in any one of claims 1 to 7 wherein R_6 combines with an NH group derived from the L_2 moiety and the terminal COOH to form an amino acid residue of the formula:

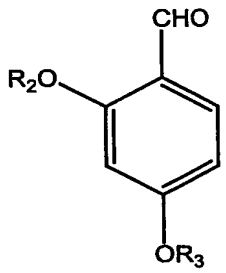
$$-NH-(CH_2)_q-Z_s-(CH_2)_rCOOH;$$

where q and r are each 0 to 3, provided that both q and r are not both 0;
s is 0 or 1; and
Z is a 5-10 membered stable monocyclic or bicyclic aromatic ring or a 3-6 membered carbocyclic or alicyclic ring.

9. A compound as claimed in claim 8 wherein r and s are 0 and q is 1 or 2.

10. A compound as claimed in any one of claims 1 to 9, which is a compound of general formula (Ia).

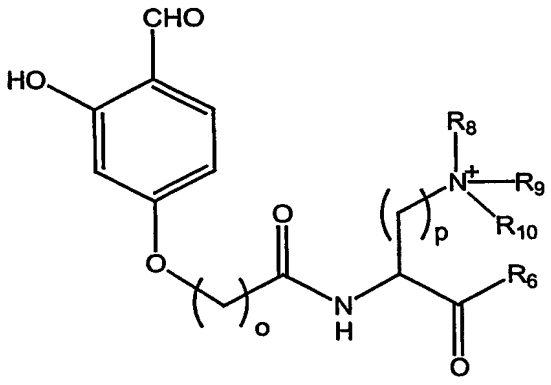
11. A compound as claimed in claim 10, which is a compound of general formula (II):



General formula (II),

which is a compound of General formula (Ia) in which X and Y are O, R_1 is H and R_2 and R_3 are as defined for general formula (I).

12. A compound as claimed in claim 11, which is a compound of general formula (III):



General formula (III)

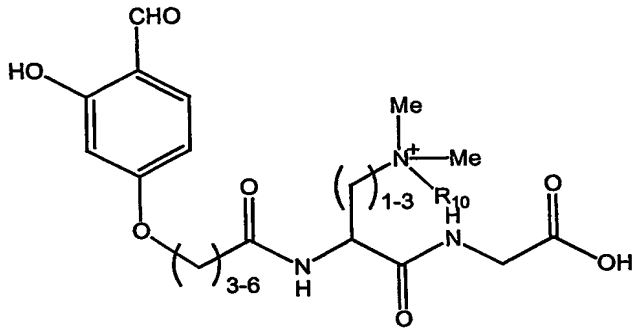
wherein:

o is an integer from 2-6;

p is an integer from 1 to 3; and

R_6, R_8, R_9 and R_{10} are as defined for general formula (I).

13. A compound as claimed in claim 12, which is a compound of general formula (IV):



General formula (IV)

wherein R_{10} = H or methyl.

14. A process for the preparation of a compound of general formula (I), the process comprising:

reacting a compound of general formula V:

$$H_2N\text{-}R_6\text{-}COOH \qquad (V)$$

wherein R_6 is as defined for general formula (I); and
wherein the compound of general formula (V) is bound at its C-terminus to a solid support;

with a compound of general formula (VI):

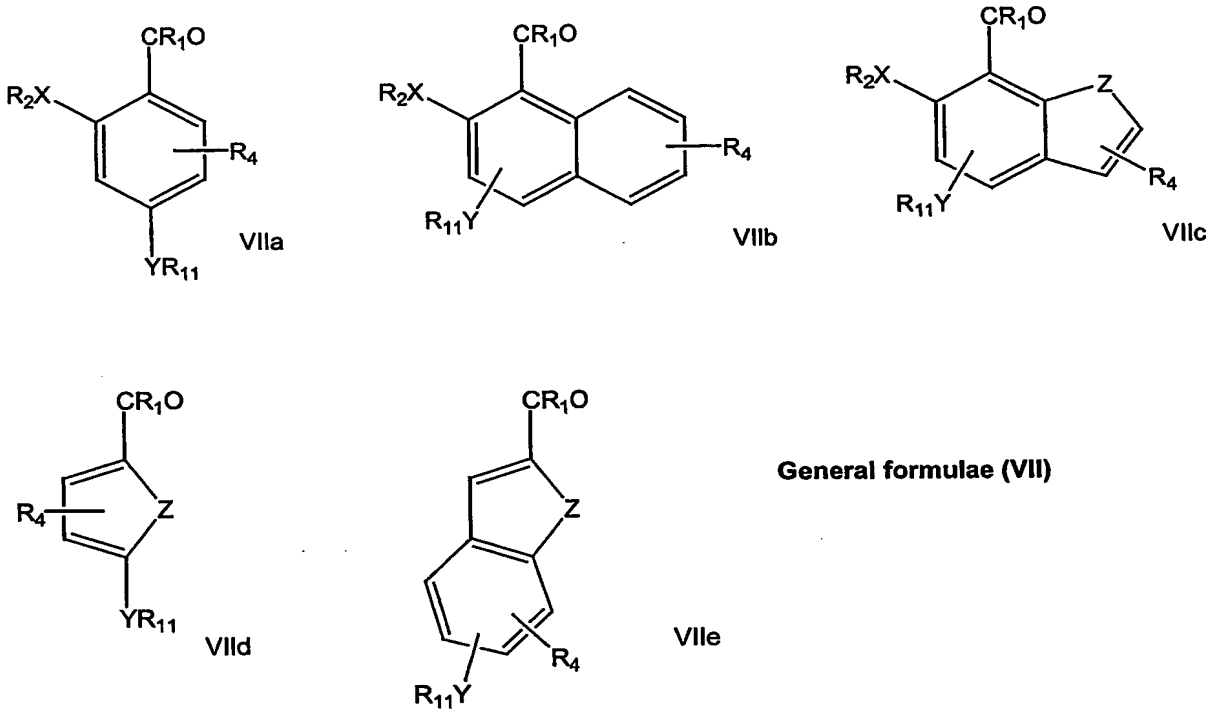
$$W\text{-}NH\text{-}R_5\text{-}COOH \qquad (VI)$$

wherein:

R_5 is as defined for general formula (I); and

W is a protecting group; and

followed by removal of the protecting group and reaction with a compound of general formula (VII)



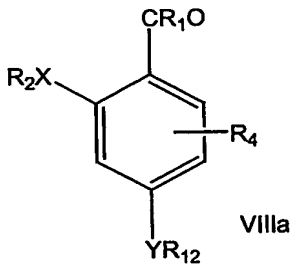
General formulae (VII)

wherein X, Y, Z, R_1, R_2 and R_4 are as defined for general formula I; and

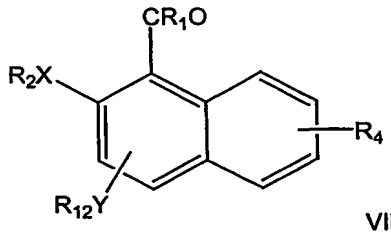
R_{11} is C_{1-7} alkyl-COOH, C_{3-10} cycloalkyl-COOH or Ar-C_{0-7} alkyl-COOH

and subsequent removal of the product from the solid support.

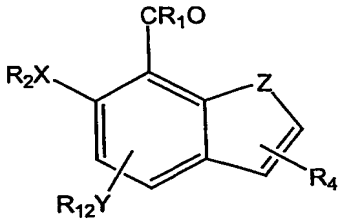
15. A compound of general formula (VIII):



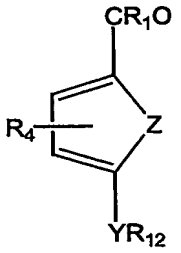
VIIIa



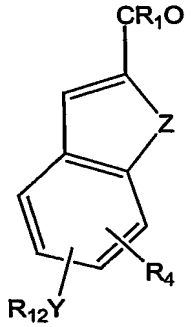
VIIIb



VIIIc



VIIId



VIIIe

General formulae (VIII)

wherein X, Y, Z, R_1, R_2 and R_4 are as defined for general formula I; and

R_{12} is C_{1-7} alkyl-L_1-R_5-L_2-R_6CONHQ, C_{3-10} cycloalkyl-L_1-R_5-L_2-R_6CONHQ or Ar-C_{0-7} alkyl-L_1-R_5-L_2-R_6CONH-Q;

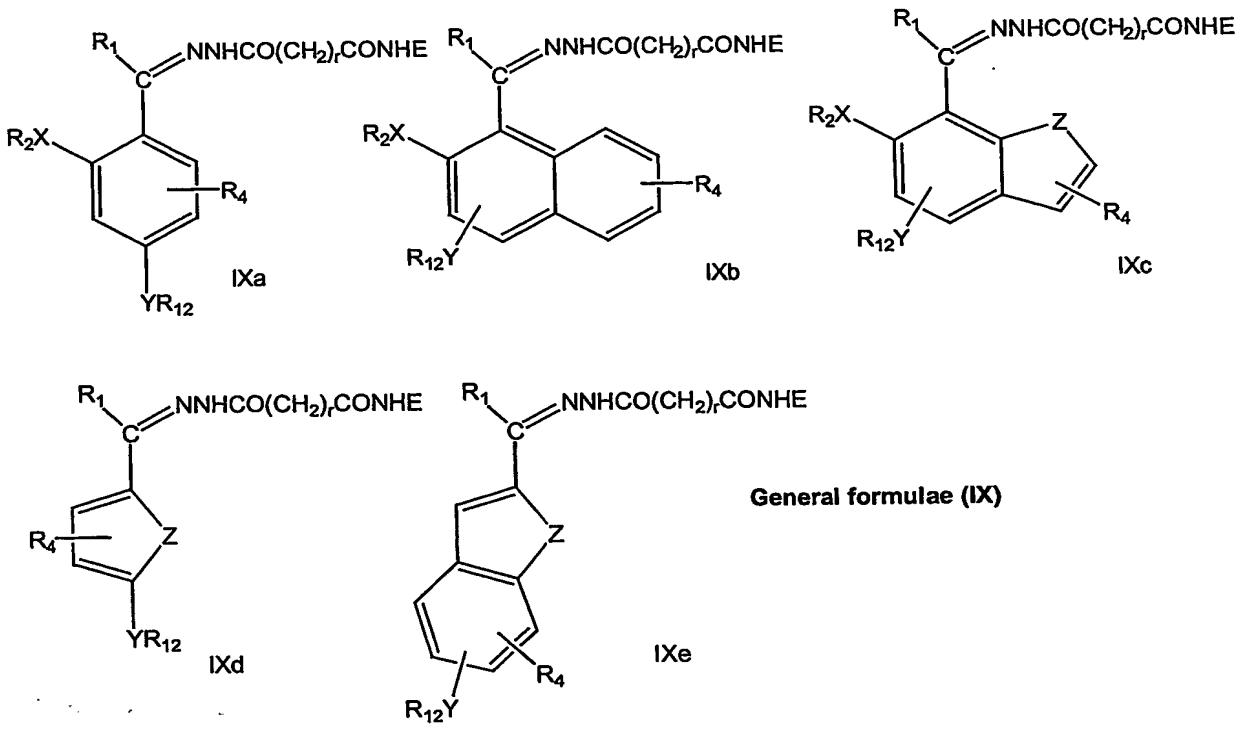
wherein L_1, L_2 R_5 and R_6 are as defined in general formula (I); and

Q is the residue of a carrier protein having a lysine side chain from which the "NH" moiety in R_{12} is derived.

16. A compound as claimed in claim 15 wherein Q is a residue of bovine serum albumen, keyhole limpet haemocyanin, ovalbumin.

17. A process for the preparation of a compound as claimed in claim 15 or claim 16, the process comprising reacting a compound of general formula (I) as defined above with a carrier protein.

18. A compound of general formula (IX):



General formulae (IX)

wherein X, Y, Z, R_1, R_2 and R_4 are as defined for general formula I;

R_{12} is as defined in general formula (VIII); and

E is derived from an epitope.

19. A compound as claimed in claim 18 wherein E is a protein or peptide molecule or a fragment, variant or analogue thereof.

20. A compound as claimed in claim 19, wherein E comprises an antigenic determinant.

21. A compound as claimed in claim 19 or claim 20, wherein E comprises oxytocin or an analogue thereof.

22. A process for the preparation of a compound as claimed in any one of claims 18 to 21, the process comprising reacting a compound of general formula (VIII) as defined in claim 15 with a compound of general formula (X):

$$E\text{-}NH\text{-}CO\text{-}(CH_2)_rCONHNH_2 \qquad (X)$$

where E is as defined in claim 18 and r is an integer from 1 to 5.

23. A compound as claimed in any one of claims 18 to 21 for use in medicine.

24. A compound as claimed in any one of claims 18 to 21 for immunising a subject in order to raise antibodies to the epitope E.

25. A compound as claimed in any one of claims 18 to 21 for use as a vaccine.

26. A pharmaceutical composition comprising a compound as claimed in any one of claims 18 to 21 together with a pharmaceutically acceptable excipient.

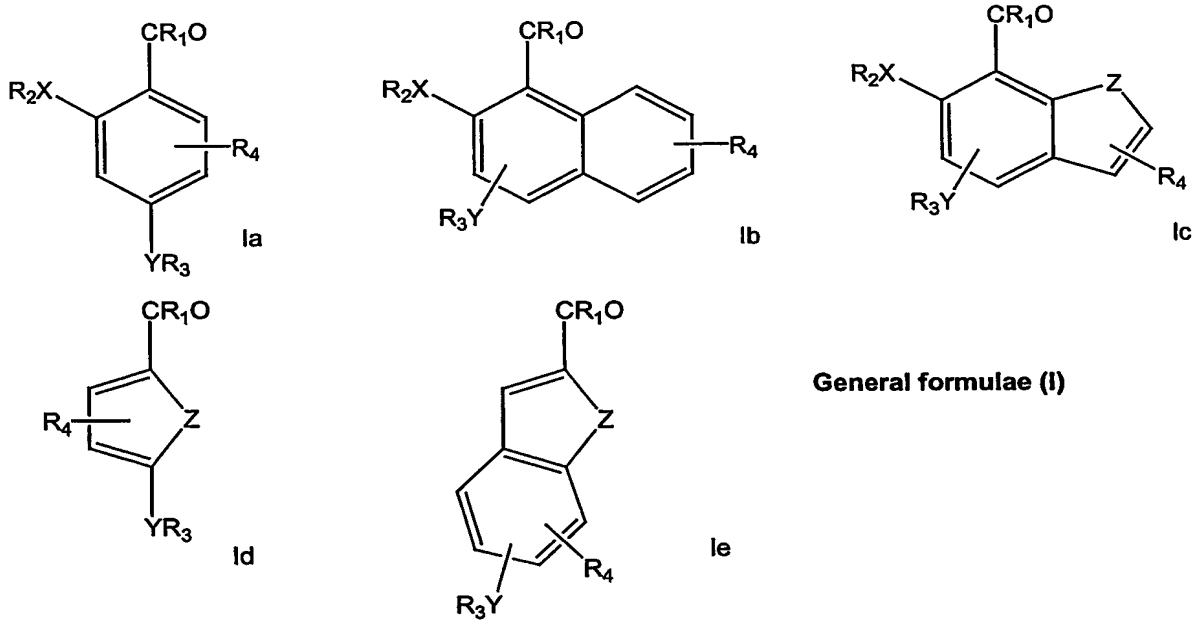
27. A vaccine composition comprising a compound as claimed in any one of claims 18 to 21 together with a pharmaceutically acceptable adjuvant.

28. The use of a compound as claimed in any one of claims 18 to 21 in the preparation of an agent for raising antibodies against the epitope E.

ABSTRACT

ORGANIC MOLECULES

Compounds according to general formulae (Ia to Ie):



General formulae (I)

wherein:

X = O or S;

Y is O, S or CHR_4;

Z is O or S;

R_1 is H or C_{1-7} alkyl;

R_2 is H or C_{1-7} alkyl;

R_3 is C_{1-7} alkyl-L_1-R_5-L_2-R_6, C_{3-10} cycloalkyl-L_1-R_5-L_2-R_6 or Ar-C_{0-7} alkyl-L_1-R_5-L_2-R_6;

R_4 is H or C_{1-7} alkyl at any vacant position on the aromatic ring;

each of L_1 and L_2 is absent or a suitable linker such as CONH;

R_5 is C_{1-7} alkyl or C_{3-6}-cycloalkyl or Ar-C_{0-7}-alkyl containing a trisubstituted nitrogen atom $NR_7R_8R_9$, or is a quaternary nitrogen atom $N^+R_7R_8R_9R_{10}$ such that the substituent R_5 contains a positively charged nitrogen atom;

R_5 is C_{1-7} alkyl, C_{3-10} cycloalkyl or Ar-C_{0-7} alkyl each of which is substituted with a trisubstituted nitrogen atom $NR_7R_8R_9$ or a quaternary nitrogen atom $N^+R_7R_8R_9R_{10}$, such that R_5 contains a positive charge;

each of R_7, R_8, R_9 and R_{10} is independently hydrogen, C_{1-7} alkyl, C_{3-10} cycloalkyl or Ar-C_{0-7} alkyl, or any two or more of R_7, R_8, R_9 and R_{10} together form an alicyclic or arylalicyclic ring system;

R_6 is C_{1-7} alkyl-COOH, C_{3-10} cycloalkyl-COOH or Ar-C_{0-7} alkyl-COOH;

and salts, hydrates, solvates, complexes and prodrugs thereof;

are of use as linkers for conjugating an epitope to a carrier protein.

FIGURE 1

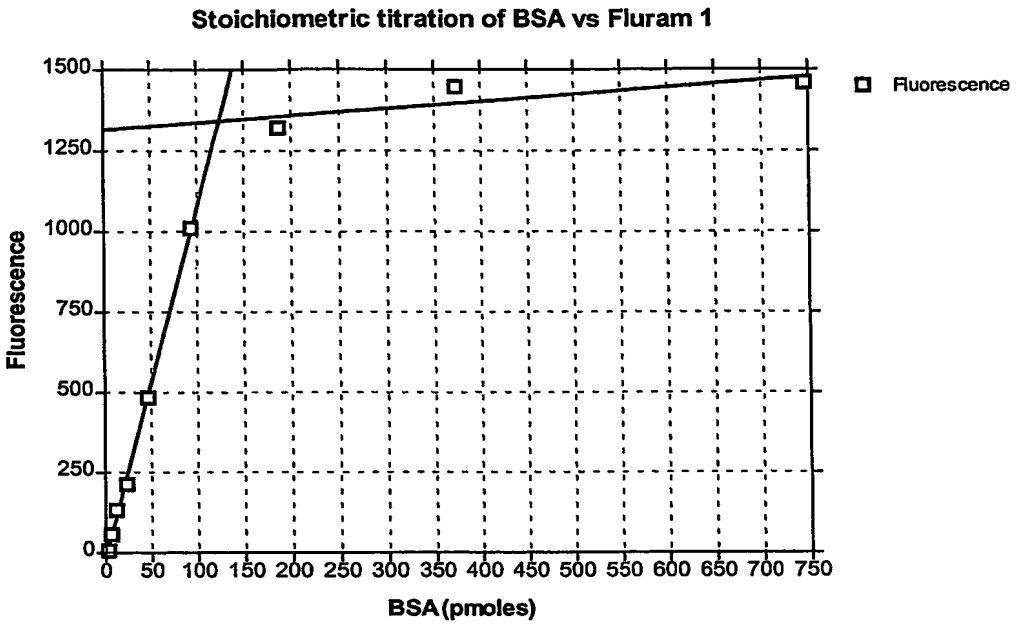


FIGURE 2

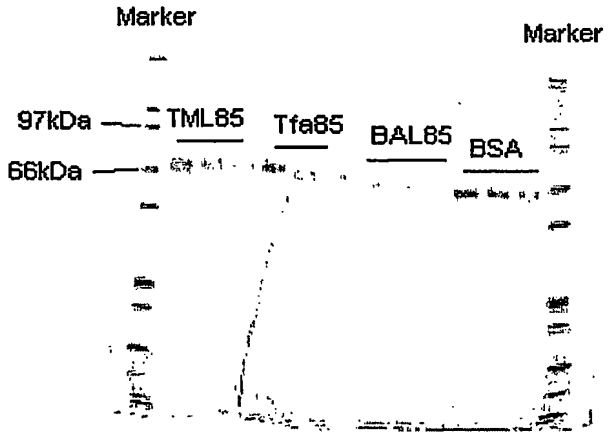


FIGURE 3

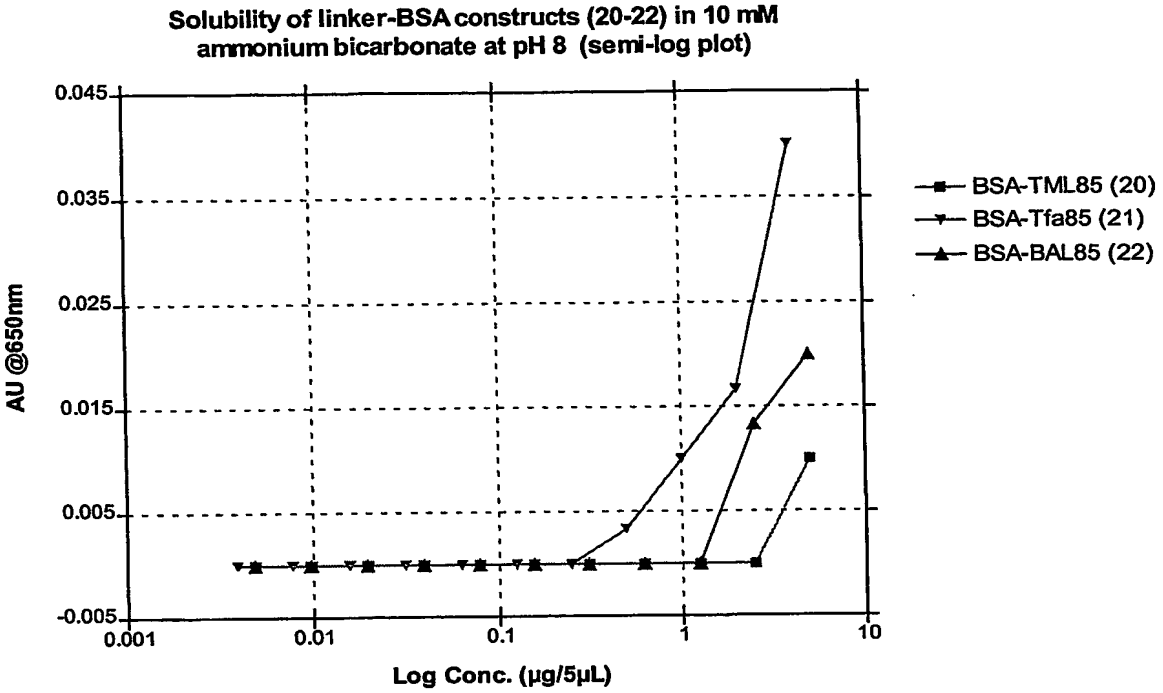

Solubility of linker-BSA constructs (20-22) in 10 mM ammonium bicarbonate at pH 8 (semi-log plot)
AU @650nm
0.045
0.035
0.025
0.015
0.005
-0.005
0.001
0.01
0.1
1
10
Log Conc. (µg/5µL)
BSA-TML85 (20)
BSA-Tfa85 (21)
BSA-BAL85 (22)

FIGURE 4

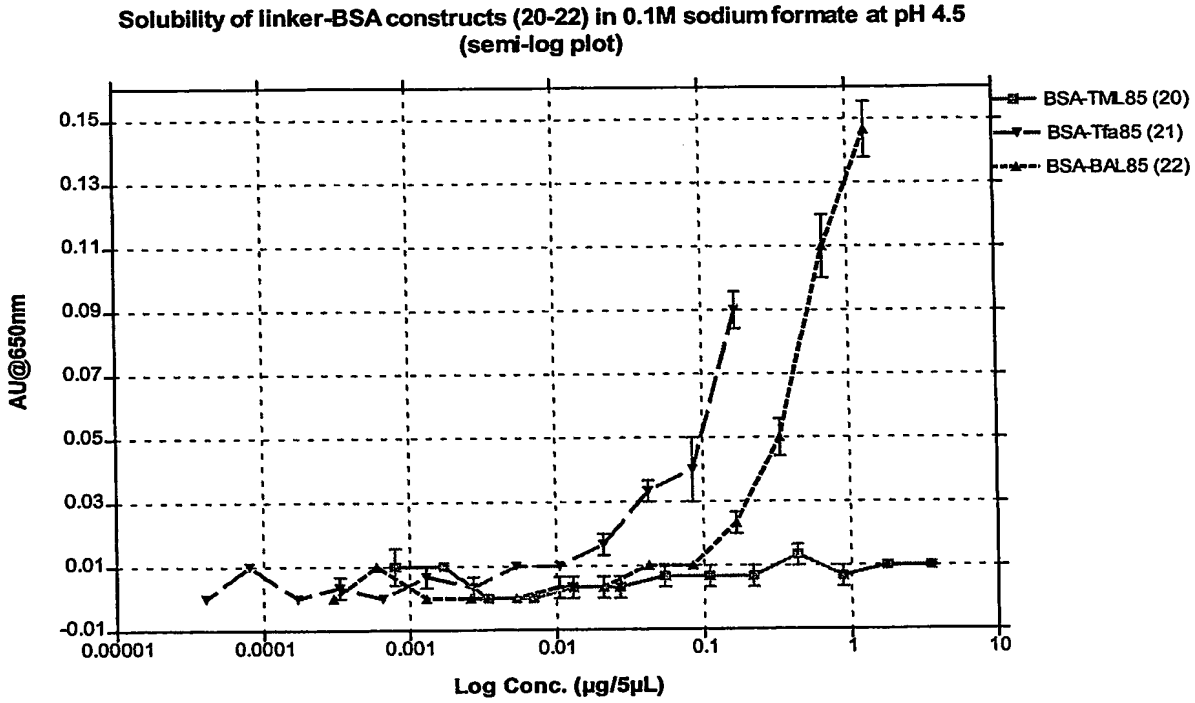

Solubility of linker-BSA constructs (20-22) in 0.1M sodium formate at pH 4.5
(semi-log plot)
AU@650nm
0.15
0.13
0.11
0.09
0.07
0.05
0.03
0.01
-0.01
0.00001
0.0001
0.001
0.01
0.1
1
10
Log Conc. (µg/5µL)
BSA-TML85 (20)
BSA-Tfa85 (21)
BSA-BAL85 (22)

FIGURE 5

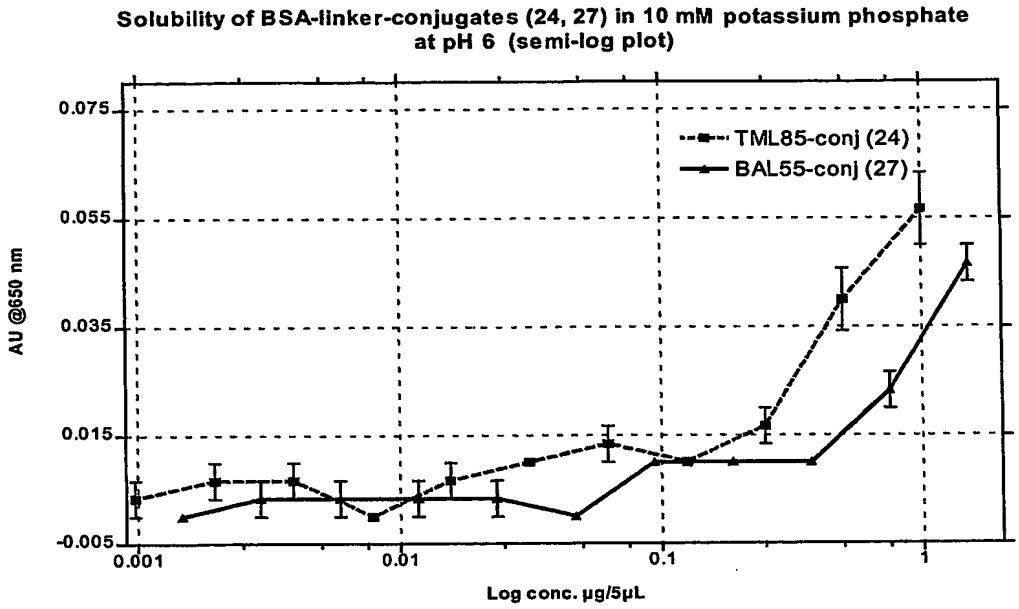


FIGURE 6

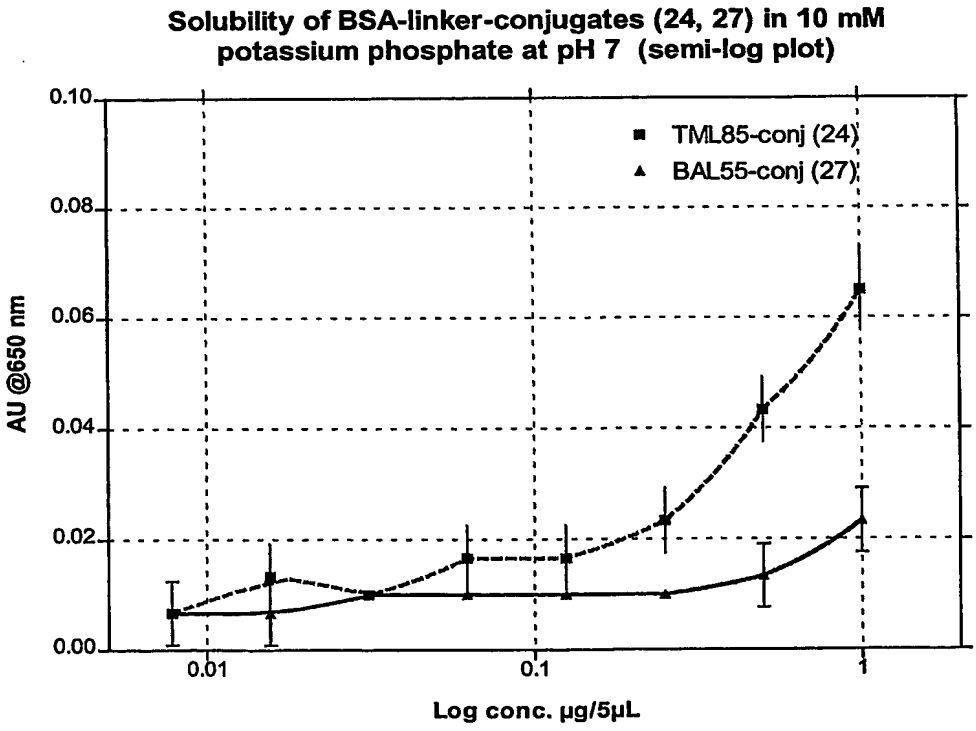


FIGURE 7

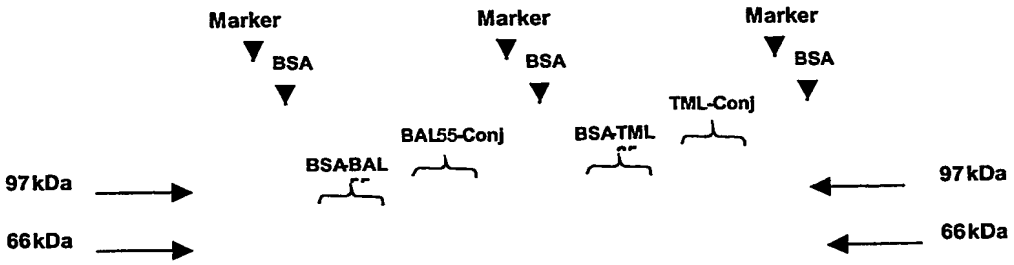


FIGURE 8

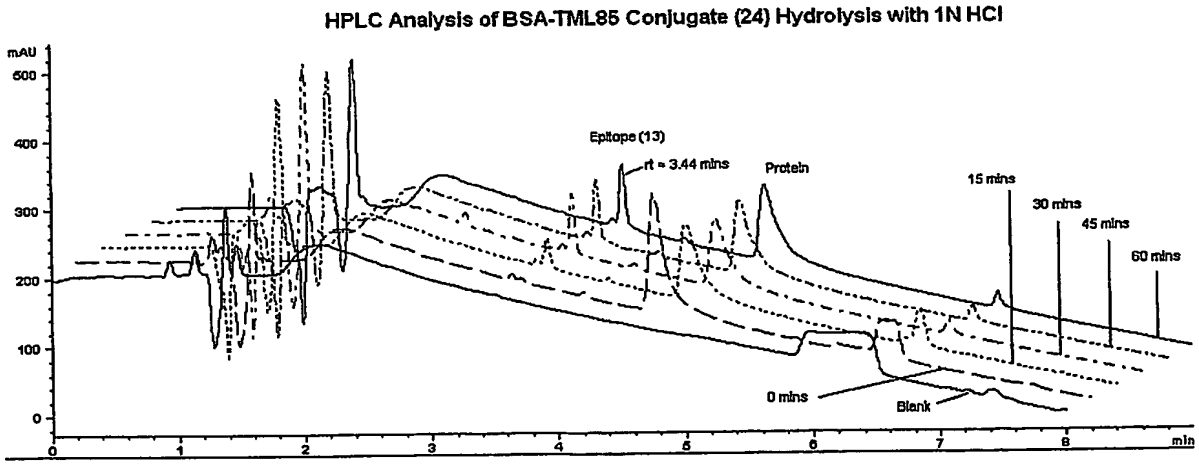


FIGURE 9

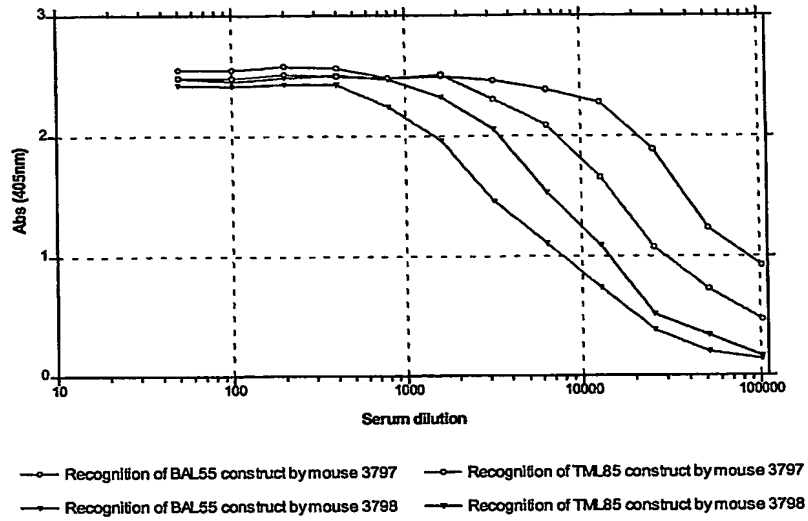


FIGURE 10

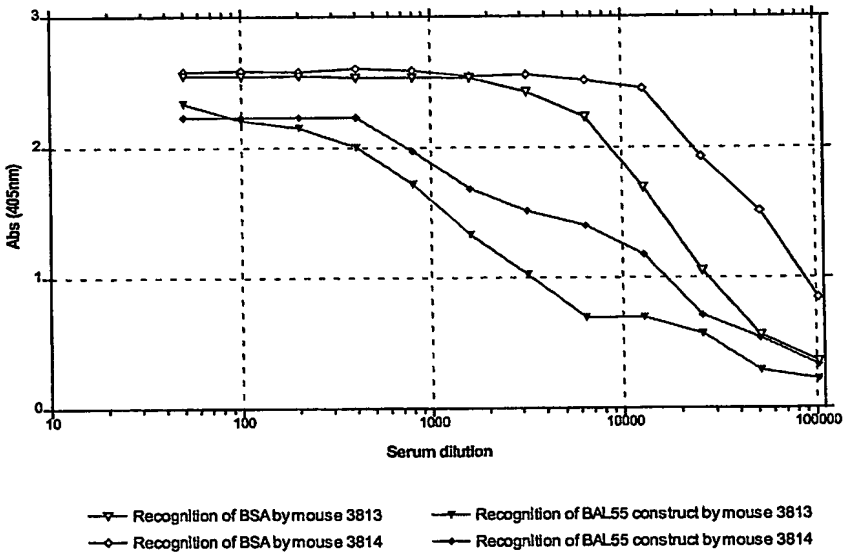
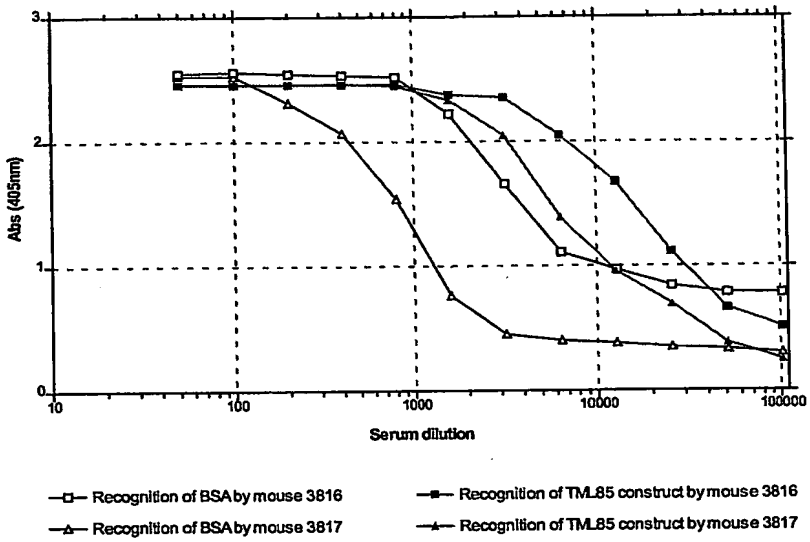


FIGURE 11


Abs (405nm)
3
2
1
0
10
100
1000
10000
100000
Serum dilution
Recognition of BSA by mouse 3816
Recognition of BSA by mouse 3817
Recognition of TM.85 construct by mouse 3816
Recognition of TM.85 construct by mouse 3817

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